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THE UNIVERSITY OF ALBERTA

ALBUMIN AND INSULIN CONJUGATES OF α-GLUCOSIDASES: PREPARATION AND KINETIC CHARACTERIZATION

BY

VIVIAN REGINA MOLINA-DE-OROZCO

A THESIS
SUBMITTED TO THE FACULTY OF
GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

OF

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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, a thesis entitled ALBUMIN AND INSULIN CONJUGATES OF α-GLUCOSIDASES; PREPARATION AND KINETIC CHARACTERIZATION submitted by Vivian Regina Molina-de-Orozco in partial fulfilment of the requirements for the degree of Master of Science in Physiology.

.....M. L. Segovia.....
Supervisor

.....R. Kasperski.....

.....J. W. Bourne.....

.....David Schiff.....

Date .....27/7/87.....

DEDICATION

TO MY COUNTRY GUATEMALA

TO ALL THE MEMBERS OF MY FAMILY

WITH LOVE

ABSTRACT

Pompe's disease is a lysosomal storage disease in which glycogen is accumulated in secondary lysosomes due to the deficiency of the lysosomal enzyme α-1-4-glucosidase.

The treatment of Pompe's disease has been attempted by earlier workers with purified α-1-4-glucosidase from A. niger and human placenta. These attempts were unfruitful due to the unstable nature of the pure enzyme and the lack of targeting. In Pompe's disease, enzyme replacement therapy will be effective only if the enzyme is delivered to those lysosomes of cells and tissues that are affected, viz., the hepatocytes and muscle cells of the cardiac and respiratory tissues. Some recent investigations have shown that the stability and targeting problems can be overcome at least partly, by cross-linking enzymes to albumin, a natural carrier, and insulin to confer targeting efficiency. However, these conjugates have not been characterized in terms of the parameters affecting their in vivo activity, an important step in evaluating their therapeutic potential.

In this project, α-glucosidase-albumin and α-glucosidase-albumin-insulin conjugates were prepared with enzymes from three different sources under different pH conditions and the suitability of these conjugates for enzyme therapy was assessed by measuring their (i) pH optimum, (ii) affinity for substrates, and (iii) thermal stability.

The results have shown that, in general, cross-linking leads to a broader pH optima, increased affinity for the substrates and better thermal stability over the native form of the enzyme. Yeast-α-glucosidase-albumin conjugates are not active towards glycogen, which is a limitation for their use in treatment of Pompe's disease where the

accumulated substrate is glycogen. Among the α-glucosidase-albumin conjugates of A. niger prepared at pH 6.8 and 4.5, the one prepared at pH 6.8 is a better polymer for replacement therapy because of its five fold increase in affinity for glycogen. α-glucosidase-albumin-insulin polymer also prepared at pH 6.8 retains the high affinity but the thermal stability is not as good as that of the albumin conjugate. The human placenta enzyme also seems to give better polymer when conjugated with albumin at pH 6.8. However, the conjugate with A. niger enzyme has a 10 fold higher affinity for glycogen compared with the human enzyme and a pH optimum closer to lysosomal pH.

The albumin conjugate of α-glucosidase from A. niger prepared at pH 6.8, thus, seems to be the best choice for enzyme replacement therapy in Pompe's disease.

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1. INTRODUCTION

1.1 ENZYME DEFICIENCY DISEASES

In 1902 Sir Archibald Garrod began his studies in alcaptonuria which were to culminate in his monograph on "Inborn errors of metabolism" (Garrod, 1909). He was able to classify a whole new set of diseases through his hypothesis of biochemical individuality and human chemical genetics. He also predicted the existence of "Enzyme Deficiency Diseases" by accurately describing a number of genetic metabolic diseases: albinism, alcaptonuria, cystinuria and pentosuria. All conditions, while not life threatening, represent alternate sources of metabolism. From his observations on these diseases he developed the concept that certain diseases of lifelong duration arise because an enzyme governing a single metabolic step is reduced in activity or missing altogether. Garrod's work was almost ignored by geneticists for a generation.

The concept mentioned above attained clear definition in the "one gene-one enzyme" principle stated by Beadle (1945) and has been well expressed by Tatum (1959) as follows:

a. All biochemical processes are under genic control.
b. These biochemical processes are resolvable into series of individual stepwise reactions.
c. Each biochemical reaction is under the ultimate control of a different single gene.

d. Mutation of a single gene results only in an alteration in the ability of the cell to carry out a single primary chemical reaction. The functional unit of DNA which controls the structure of a single polypeptide chain is frequently called a cistron and the one gene-one enzyme principle has been redefined to the one cistron-one polypeptide concept. A proposed modification of the fourth item of Tatum's statement (Stanbury et al, 1983) is:

e. Mutation of a structural gene causes a change in primary structure of a specific protein and may also affects the quantity of a limited number of other proteins. Mutation of a control gene alters the extent of function of one or more structural genes and therefore alters the amount of one or more proteins without changing their structure. Mutations of other types of genes may have a variety of complex effects, difficult to classify at present.

The term molecular disease was introduced by Pauling et al, (1949) in their original paper on the abnormal electrophoretic behavior of sickle-cell haemoglobin to describe a disease in which a structural alteration in a macromolecule had led to a specific functional change which was responsible for the disease state.

The consequences of a genetic alteration in quality or quantity of a protein will depend on the role normally served by that protein (Stanbury et al, 1983). The potential consequences are:

a. Failure of formation of a specific product. In von Gierke disease absence of glucose-6-phosphatase activity leads to hypoglycemia on fasting.

b. Accumulation of precursors of the blocked reaction. If the accumulated metabolites are soluble their concentration in body fluids will be raised and their excretion in urine increased. If the accumulated metabolites are poorly soluble they may be stored. The thesauroses or storage diseases fall largely in this category. Among these are disorders of lipid storage, mucopolysaccharidoses and glycogen deposition diseases.

c. Overproduction diseases. Attributable to excess activity of a regulatory enzyme. Example: phosphoribosylpyrophosphate synthetase overactivity associated with purine overproduction and gout.

d. Transport diseases. For example cystinuria, iminoglycenurias and renal glycosuria. In these disorders a specific membrane transport protein is thought to be deficient.

e. Receptor disorders. Specialized receptors exist on cell membranes, in cytoplasm and in nuclei, for binding of many biologically active molecules prior to the exhibition of their regulatory functions. For example, type 2 hyperlipoproteinemia has been shown to be associated with specific deficiency of the LDL receptors on the cell membrane.

The complexity of enzyme deficiency diseases may not be in the Substrate or product accumulation that causes a problem but in the implications that it can have in the general metabolism. Such is the case of phenylketonuria. In classic phenylketonuria, the activity of phenylalanine hydroxylase is almost totally deficient. Phenylalanine hydroxylase hydroxylates phenylalanine to tyrosine which is utilized in

the synthesis of melanin, epinephrine and thyroxine. This hydroxylation is limited to the kidneys, liver and pancreas in mammals. Classic phenylketonuria is inherited as an autosomal recessive trait and is widely distributed among caucasian ethnic groups and orientals. It is rare in blacks. Phenylalanine accumulation in blood and urine and reduced tyrosine formation are direct consequences of the impaired hydroxylation. In untreated phenylketonuria plasma concentrations of phenylalanine become sufficiently high to activate alternate pathways of metabolism and lead to formation of phenylpyruvate, phenylacetate, phenylactate and other derivatives that are rapidly cleared by the kidney and excreted in urine. Plasma concentrations of several other aminoacids are moderately reduced, probably secondary to inhibition of gastrointestinal absorption or impairment of renal tubular reabsorption by the excess phenylalanine in body fluids. The severe brain damage observed in untreated phenylketonuria appears to be related to several consequences of phenylalanine accumulation: deprivation of other aminoacids required for protein synthesis, impaired polyribosome formation or stabilization. Reduced myelin synthesis and inadequate formation of norepinephrine and serotonin. Phenylalanine is a competitive inhibitor of tyrosinase, a key enzyme in the pathway of melanin synthesis. This block plus reduced availability of the melanin precursor, tyrosine, accounts for the hypopigmentation of hair and skin (Petersdorf et al, 1983).

In lysosomal storage diseases the accumulation of metabolites occurs in the lysosomes. The name lysosomes (lytic bodies) was given by de Duve and co-workers in 1955 to a group of cytoplasmic particles that contain hydrolytic enzymes. In a freshly prepared liver

homogenate these enzymes are separated from the surrounding medium by a membrane of lipoprotein nature that restricts their accessibility to external substrates (Hers, 1965). Some 40 enzymes are now known to be contained in lysosomes. They are all hydrolytic enzymes including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases. And all are acid hydrolases, optimally active near the pH of 5 (Alberts et al, 1983). Lysosomes have diversity of shapes and sizes and are found in all eucariotic cells. The heterogeneity of lysosomal morphology contrast with the relative uniform ultrastructure of all other cellular organelles. This diversity reflects the wide array of different digestive functions mediated by acid hydrolases, including the digestion and turnover of intra- and extra-cellular constituents, programmed cell death in embryogenesis, digestion of phagocytosed micro-organisms and cell nutrition (cholesterol assimilation from endocytosed serum lipoprotein) (Alberts et al, 1983).

In 1963, Hers discovered the glycogen deposits of type II glycogen storage disease were not found free in the cytoplasm as in other glycogenoses, but rather were localized within lysosomes. He defined a lysosomal deficiency disease as one in which: 1) a single lysosomal enzyme is deficient, and 2) abnormal deposits of substrates lie within membrane bounded vesicles.

Hers (1965) made a number of predictions about Inborn Lysosomal Diseases:

a. As a general morphological symptom, one should find abnormal cellular inclusions consisting in large vacuoles derived from the lysosomal system and therefore, possibly showing a positive

reaction for acid phosphatase, lined by a single membrane, and filled with undigested material or residues; the usual forms of lysosomes would have disappeared, but intermediary figures between them and the vacuoles might be encountered.

b. The accumulated material could be chemically homogeneous and present the same appearance in all affected cells; but this need not necessarily be so, since a single hydrolase may be involved in the digestion of numerous substances. If the material stored in the lysosomes does not appear in blood, autophagy is mainly responsible for its uptake. When the material occurs in the extracellular environment, endocytosis may also become involved.

c. Like other genetic defects, inborn lysosomal diseases are expected to extend to all the cell types in which the missing enzyme is controlled by the same gene. However, the manifestations of the diseases may vary greatly in intensity from one afflicted cell type to the other, since they are governed by the rate at which the indigestible material accumulates in the lysosomes, by endocytosis or autophagy, by the excretory possibilities of the cell, and also by their lifespan. Short lived cells, such as the polymorphonuclear leukocytes and the epithelial cells may not have the time to accumulate detectable amounts of the material.

d. An important characteristic of inborn lysosomal diseases is their progressivity. Patients with such diseases may appear essentially normal at birth; they will develop the symptoms of the disease progressively, at a rate which depends on the nature and abundance of the stored material and on its preferential site of accumulation. Type II glycogenosis, which is usually fatal in

less than one year, may not be typical in this respect, and other diseases involving the slow accumulation of a rare material may require years to manifest themselves clinically.

e. The correlation between the basic lysosomal alterations and the clinical manifestations of the disease may be difficult to establish. Mechanical interference with the normal functioning of the cell, disruption of the lysosomal membrane or functional deficiency of the lysosomes are possible events. Whereas many different cell types may exhibit signs of lysosome enlargement, eventually reflected in gross anatomical changes, such as hepatomegaly or splenomegaly, the functional symptoms are expected to be specific of the site where the condition first ceases to be tolerated. The expansion possibilities of each tissue may be a point of importance to this respect.

f. There exists a theoretical basis for enzyme replacement therapy for diseases of this type.

1.2 TREATMENTS

More than a hundred years ago Purdon (1871) demonstrated the use of proteolytic enzyme pepsin during a number of surgical procedures for controlling and cleaning abscesses, varicose ulcers and ulcerating cancers to inhibit bacterial growth and other contamination.

By 1960, while enzymes were not yet in any important widespread clinical use, their potential was being investigated for the treatment of a wide range of enzyme deficiency diseases, as antineoplasic agents and as a means of controlling enzymatic activation of fibrinolisis in the treatment and/or control of tromboembolic vascular disease (Poznansky, 1983).

The problems associated with enzyme therapy (Poznansky, 1983) are:

a. Introduction of exogenous proteins into the circulation often results in their rapid degradation by proteolysis. This may necessitate repeated administration of the enzyme.

b. The degree of purification of the enzyme has to be considered. The purification of sufficient quantities of enzyme for enzyme replacement therapy is a serious limitation.

c. The highly immunogenic nature of most enzyme preparations currently available as therapeutic agents. Typical enzymes available in sufficient quantities have been derived from bacterial or fungal sources and resulted in moderate to severe hypersensitivity reactions.

d. The site of substrate accumulation and hence the route of administration of enzymes is another drawback to the common

use of enzyme therapy. The enzyme must be administered in such a way that it has access to the accumulated substrate.

In Pompe's disease, where glycogen accumulates and remains in secondary lysosomes, enzyme replacement will be effective only if the enzyme is delivered to those lysosomes of cells and tissues that are affected. In this case, hepatocytes and muscle cells of the cardiac and respiratory tissues must be the target cells. In Tay-Sachs disease, where the central nervous system is the more affected tissue by substrate accumulation, the drug must cross the blood-brain barrier.

Macromolecular drug carrier systems have been developed in an attempt to alter the tissue localization of drugs such that the effects of drugs at desired sites of action are enhanced relative to effects at sites at which drug effects are unwanted. Macromolecular drug carriers have also been applied to the problems of delivery and bioavailability in enzyme replacement therapy. In this case, the carrier may have an important action in reducing the antigenicity of a heterologous enzyme as well as improving delivery of the enzyme to its desired site of action. A variety of carrier systems have been used in both animal and clinical experiments (eg. antibodies, albumin, lipoproteins, dextrans and synthetic macromolecules such as polyethylene glycols and polylysines) (Poznansky and Cleland, 1980). According to these authors, there are certain characteristics which a drug carrier must possess:

a. The carrier-agent conjugate must retain the agent's activity unless the complex can be degraded at the site of desired action with the release of the agent in its active form.

b. The carrier must be without intrinsic toxicity.

c. The carrier must be non-immunogenic, non-antigenic and should not adversely alter the antigenicity of the compound for carriage.

d. The carrier must be biodegradable.

e. The carrier must retain its own desirable characteristics following conjugation with a drug or enzyme.

The following objectives may be achieved using drug or enzyme carrier systems (Poznansky and Cleland, 1980):

a. Stabilization of the drug or enzyme in its active form, thereby retarding normal biodegradation.

b. Improved localization of agent at sites of desired action.

c. Increased circulation half time. This is of particular importance for agents whose action involves detoxifying or reducing substrate levels in the plasma. Delayed plasma clearance may also improve bioavailability of agents having an extravascular action.

d. Alteration of drug or enzyme solubility. For example, a lipophilic drug or enzyme for parental administration could be made water soluble by linkage with a more hydrophilic carrier.

e. Reduction of immunogenicity and antigenicity of enzymes.

As mentioned earlier, a wide number of carrier systems have been used in vivo and in vitro including liposomes, antibodies, albumin, low density lipoproteins, fibrinogen, collagen, dextrans, hormones,

synthetic biopolimers, etc. and, of these, albumin seems to be the most promising one.

The advantages of albumin as a drug or enzyme carrier system (Poznansky, 1985) are:

a. Albumin is a natural and the most abundant plasma protein.
b. Albumin has a relatively long circulation time, with a turnover rate in the order of 30 hours.
c. Albumin probably functions normally as a carrier molecule, being responsible for the carriage of fatty acids as well as both steroid and polypeptide hormones in the plasma.
d. Albumin is relatively stable, is inexpensive and readily available and it has a multiple of reactive sites on which to attach therapeutic agents.

In 1974, Palliot et al (1974) described some in vitro properties of several soluble, cross linked enzyme polymers. The main advantage of such a procedure is that it immobilizes the enzyme in a soluble form, and the preparation may have easier accessibility to the substrate while remaining in the circulation. They demonstrated the covalent binding of an excess albumin with either hog liver uricase or E. coli L-asparaginase using glutaraldehyde as the cross linking agent (to produce a Schiff base linkage) to form a soluble polymer with increased resistance to heat and proteolytic denaturation over the naked or free enzyme. The method provides a great deal of versatility in controlling the size of the complex, the ratio of enzyme to inert protein and the ability to work with fragile systems.

Poznansky (1977) continued the studies on the enzyme uricase to examine resistance to heat at 37°C and pH characteristics of the free and albumin conjugated forms. These studies showed that conjugation increases heat stability at 37°C and by altering the pH of the buffer at the cross-linking step, the pH optimum was altered from a sharp peak at pH 9.6 for the naked enzyme to a broad peak between pH 8.0 and 9.6 for the immobilized enzyme.

In 1978, Remy and Poznansky showed that polymeric complexes of hog liver uricase and rabbit albumin were non-immunogenic in rabbits (they did not elicit an antibody production) and non-antigenic (antibodies against hog liver uricase did not react with the polymeric structure). The control showed that the uricase, cross-linked with dog albumin, elicited an antibody response in rabbits against the polymer as a whole and against the dog albumin but not against the hog liver uricase, whose antigenic determinants (it is highly immunogenic in its native form) must therefore have been masked. These have been corroborated in further studies with other enzymes: yeast α-1-4-αglucosidase, human placenta α-1-4-glucosidase, bovine superoxide dismutase and L-asparaginase (Poznansky, 1984).

The molecular weight of the enzyme-albumin polymers is a function of the cross-linking agent used (eg. glutaraldehyde) and the length of time the conjugation reaction is allowed to proceed. α-glucosidase-albumin polymer has a molecular weight between 7.5×10^5 and 8.5×10^5 suggesting that the average molecule of polymer contains approximately 12 molecules of albumin per enzyme molecule) (Poznansky and Bhardwaj, 1980).

The retention of enzyme activity following conjugation for yeast α-glucosidase was 70% of the original activity and for placenta-α-glucosidase was 80 to 85% (Poznansky and Singh, 1982). Poznansky (1979) used uricase-albumin polymer to reduce the uric acid levels of Dalmatian coach hounds, a canine breed that suffers chronic hyperuricemia, observing that the enzyme-albumin complex remained in the circulation for approximately five times longer than equivalent amounts of the free enzyme (half life of 20 hours opposed to 4 hours) and was more effective in lowering plasma uric acid levels.

As mentioned earlier, the net result of lysosomal storage diseases is a gross accumulation of secondary lysosomes packed with undegraded substrate, altering severely the cellular and tissue function. Then, consideration of enzyme replacement therapy for these diseases requires specific delivery of the enzyme not only to specific tissues and cells but to the secondary lysosomes in which substrate accumulates. In Pompe's disease there is hepatosplenomegaly due to accumulation of glycogen in liver and spleen. But the critical site of glycogen accumulation is the cardiac and respiratory muscle which is the cause of cardiorespiratory dysfunction and eventual death.

The problem is to target the enzyme to these muscular tissues. Poznansky and Singh (1982) decided to use insulin as a targeting agent. Muscle cells have a very high density of insulin receptors. They conjugated insulin to α-glucosidase-albumin polymer, resulting in an enzyme-albumin-insulin polymer of molar ratio averaging 1:12:60 with an average molecular weight of 1.2×10^6. They obtained four lines of evidence to indicate that insulin is conjugated to the enzyme-albumin polymer:

a. Anti-insulin antibodies react with enzyme-albumin-insulin polymers but not with enzyme-albumin conjugates.

b. Enzyme-albumin-insulin conjugates are cleared from the circulation with a half time of 4 hours as compared to 16 hours for the enzyme-albumin complex alone.

c. Enzyme-albumin-insulin polymers retain the hypoglycemic effect of insulin and roughly the same glucose lowering ability that an equivalent amount of free insulin might be expected to produce.

d. Enzyme-albumin-insulin conjugates bind preferentially to mouse spleen cells and to chick embryonic muscle cells, both in tissue culture. Preliminary data in vivo indicate that the insulin conjugate targets preferentially to tissues bearing high densities of insulin receptors (Poznansky, 1983).

1.3 POMPE'S DISEASE

In 1932, Pompe in Holland and Bischoff and Putschar in Germany, independently described patients dying in infancy with an enormous enlargement of the heart due to diffuse deposition of glycogen in this organ (di San't Agnese et al, 1950). Subsequently, this condition was called cardiomegalia glycogenica, Pompe's disease or glycogenosis Type II, as well as cardiomuscular and generalized glycogenosis, since glycogen deposits were found in various organs.

In 1963, Hers found that α-1-4-glucosidase (acid maltase) was deficient in patients with the disease. The enzymatic defect is inherited as an autosomal recessive trait, and sibships with more than one affected sibling have been reported (Smith et al, 1967).

Three clinical presentations have been described:

1.3.1 INFANTILE FORM:

Symptoms begin between the second and sixth month of life but may be present from birth. Vomiting, anorexia, failure to grow, weakness of the musculature, drooling and later cyanosis and dyspnea are the most frequent symptoms and signs (Stanbury et al, 1960).

Physical findings may include:

a. Appearance of imbecility not unlike that seen in cretinism or mongolian idiocy at times enlargement of the tongue occurs.

b. Profound hypotonia during the first year of life. The muscles are firm and of normal mass.

c. Enlargement of the heart can usually be detected by percussion and palpation and an apical systolic murmur is not uncommon.

d. Hepatosplenomegaly in some cases.

Laboratory examinations reveal:

a. Fasting blood sugar, ketone concentration, glucose tolerance, galactose tolerance, glucagon and epinephine responses are all normal.

b. a globular cardiac silhouette is a common finding in roentgenographic examination of the chest.

c. the electrocardiogram shows the specific changes of gigantic QRS complexes in all leads and a shortened P-R interval.

The central clinical problem is cardiac failure with tachycardia and edema. In a few cases death ensues from aspiration pneumonia and rogressive weakness of the muscles of respiration. Death from one or another mechanism within the first year is the rule (Stanbury, 1960, 1983).

The autopsy findings are:

a. A massive increase in normally structured glycogen in most tissues. Increased glycogen concentrations are found in muscle, liver, heart and tongue.

b. The most extensive deposition in the central nervous system is in the motor nuclei of the brainstem and anterior horn cells of the spinal cord, only slight deposition occurs in the cortical neurons.

c. Although there is increased glycogen in the Schawn cells of peripheral nerves, no dysfunction occurs.

d. About one fifth of the patients have endocardial thickening compatible with a diagnosis of endocardial fibroelastosis (Stanbury et al, 1983).

1.3.2 CHILDHOOD FORM:

The disease appears in infancy or early childhood and progresses much more slowly than the infantile form. Organ involvement is variable. No patient has survived beyond 19 years.

The following signs and symptoms characterized this form (Smith et al, 1967): delay of motor development, difficulty to walk, difficulty in swallowing, lumbar lordosis, winged scapulae and a flattened toraxic cage, thin extremities with muscles somewhat atrophic. The disease progresses rapidly in the last years of life, respiration becomes mainly diaphragmatic until death occurs.

Normal laboratory findings were:

a. Fasting blood sugar, glucose tolerance, galactose tolerance, response of glucose to epinephrine and glucagon were normal.

2. Electrocardiogram and chest X-rays were normal.

1.3.3 ADULT FORM:

Patients with the adult form do not present organomegaly but are marked clinically by muscular weakness mimicking other chronic myopathies. The biochemical findings are similar to those found in other forms of the disease (Stanbury, 1983).

The diagnosis of Pompe's Disease is established by the demonstration of an increased tissue concentration of glycogen in association with an α-1-4-glucosidase deficiency.

Attempts to treat Pompe's Disease using enzyme replacement therapy has been disappointing (Huijing et al, 1973; de Barsy et al, 1973; Williams and Murray, 1979).

1.4 α-1-4-GLUCOSIDASE (ACID MALTASE)

The nonphosphorolytic breakdown of glycogen and maltosyl oligosacharides in mammalian tissues can take place through the breaking up of the chains into fragments several glucose units long or by liberation of one glucose at a time. These glucosidic fragments can be transferred either to water (hydrolysis) or to specific acceptors (transglucosylation) (Torres and Olavarria, 1961).

Hydrolysis of glycogen in liver and muscle to give polyglucosidic fragments is performed by α-amylase. Two enzymes liberate only glucose from the polysacharide molecule α-glucosidase and amylo 1-6-α-glucosidase, which is active on glycogen limit dextrins. The maltosyl oligosaccharides can be degraded by α-amylase or, preferentially, by glucosidase (Torres and Olavarria, 1961).

α-1-4-glucosidase sediments during cell fractionation in the lysosome rich light mitochondrial fraction (Lejeune, 1963). The enzyme hydrolyses maltose and glycogen into glucose and catalyses transglucosylation from maltose to glycogen (Lejeune, 1963). The lysosomal location of acid maltase was confirmed by density equilibration in a density gradient, by its structure linked latency and by its release under controlled damage (Lejeune, 1963). It has been suggested that the enzyme possesses three binding sites: one for maltose and other oligosaccharides, one complicated in transglucosylation and the third binds polysaccharides such as glycogen (Palmer, 1971). Hers (1963) found that α-1-4-glucosidase was absent in tissues from Pompe's disease patients.

α-glucosidases from different sources have been characterized and a partial list is shown in Table 1.4.1.

TABLE 1.4.1

CHARACTERISTICS OF α-GLUCOSIDASES FROM DIFFERENT SOURCES

SOURCE OF -GLUCOSIDASE	pH	Km	INHIBITION	OTHER CHARACTERISTICS	REFERENCE
Rat liver	optimum between 4 and 5	5×10^3 M with maltose		Releases glucose from glycogen and malto-sligosacharides.	Torres and Olavarria (1961)
Rat liver	acid: 4 with maltose neutral: between 6 and 7 with maltose		acid: with turanose 50% at 5mM and 100% at 30 mM	acid: found to be bound to lysosomes.	Lejeune et al (1963)
Dog liver	pH range 3 to 6 (acid) 4 to 7.5 (neutral)	4-5 mM maltose (acid) 16 mM glycogen .8-1.4 maltose (neutral) 25 mM glycogen		acid: degrades practically all α-glucosyl disacharides, glycogen, and α-glucosides. Neutral: most specific specific towards maltose. Both showed glucosyltransferase activity. Acid: localized in lysosomes; Neutral: in supernatant fluid.	Torres and Olavarria (1964)
Cattle liver	optimum 4.5 with maltose and glycogen	$1x10^{-2}$ M (maltose) 28 mg/ml (glycogen)	inhibited by turanose, erythritol and Tris	activity as a function of temperature; stable at up to 50°C for 15 minutes. M.W. 107,000.	Bruni et al (1969)
Rat liver	pH 3.7 for maltose pH 4.4 for glycogen	3.8 mM (maltose) from .7 mM to 6.5 mM (glycogen)	Maltose inhibits glycogen hydrolysis; Glycogen inhibits maltose hydrolysis	Lysosomal enzyme with 1-4 and 1-6 activities. Can act as a transglucosylase. M.W. 80,000 to 114,000 with sucrose density centrifugation 107,000 by equilibrium sedimentation.	Bruni et al (1970)
Cattle liver	optimum 5 with maltose and glycogen (for Transglucosilation)			Disassociated with guanidine HCl not with alkaline pH or acylation given a sedimentation constant of 1.8 S compared to native enzyme 5.7 S. Its suggested enzyme is a molecule composed of subunits of similar molecular weight held together by non covalent inter-actions.	Bruni et al (1970)
Rabbit muscle	4.5 with maltose 5.1 with glycogen	3.7 mM (maltose) 2.4 mg/ml (glycogen)	by more than 5 mM of maltooligosacharides by Iodoacetate (for glycogen)	0.1 M NaCl stabilized the enzyme and increased concentrations of NaCl increased the activity of the enzyme using maltose and glycogen. Enzyme exhibits α-1-4-glucosidase, α-1-6-glucosidase and glucoamylase activity. It catalizes the complete conversion of glycogen to glucose. The suggested acid-α-glucosidase may possess 3 specific substrate binding sites: 1) for maltose and other oligosaccarides, 2) complicated in the nucleophilic attack on enzyme-glucosyl intermediary complex, 3) binds polisaccharides such as glycogen.	Palmer (1971)
Human placenta	optimum 4 for maltose and glycogen	11 mM (maltose) 2 g/ml (glycogen)	by turanose, by specific antibodies	Absence of inhibition by excess maltose.	de Barsy et al (1972)
Human liver	optimum 4 for maltose optimum 4.5 for glycogen	8.9 mM (maltose) 9.6 mM (2.5%) for glycogen	by specific antibodies, more effective on glycogen than maltose.	Protein composed of 2 subunits with M.W. 88,000 and 76,000. Estimated M.W. 110,000. No substrate inhibition by maltose up to 80 mM. Considerably higher specific activities at saturating concentrations of maltose, glycogen and isomaltose.	Murray et al (1978)
Human heart	optimum 4 for maltose optimum 5 for glycogen	5 mM (maltose) 8 mM (glycogen)	inhibition by cations copper and iron	molecular weight of 111,000 daltons. No substrate inhibition by maltose.	Chambers & Williams (1983)

1.5 SCOPE OF THE PRESENT WORK

From the foregoing discussion, it is clear that an effective enzyme replacement therapy for Pompe's disease requires α-glucosidase conjugates containing a suitable carrier molecule to stabilize it in circulation and a targeting agent which will direct the enzyme to the tissues where glycogen is accumulated. The studies so far have shown that albumin is an ideal carrier and insulin is a potential targeting agent. The next step towards developing a successful enzyme replacement therapy is the manipulation of the cross-linking step by adjusting parameters such as the ratio of different proteins, pH of the reaction medium and so on to obtain a conjugate with optimum activity under *in vivo* conditions. This, in turn, requires a characterization of various conjugates in terms of factors affecting their *in vivo* activity viz. the pH optimum for α-glucosidase activity, the affinity for different substrates and thermal stability. Thus, a project was undertaken to prepare α-glucosidase-albumin and α-glucosidase-albumin-insulin conjugates under different pH conditions and to assess the suitability of these conjugates for enzyme replacement therapy by measuring their (i) pH optimum, (ii) affinity (Km) for substrates, and (iii) thermal stability. Enzymes from two non-human sources were also considered to see whether these have any advantages over the human enzyme despite the possibility of unfavourable immunological reactions.

2. MATERIALS AND METHODS

2.1 METHODS

2.1.1 Enzyme Assay

α-glucosidase activity was measured by monitoring the release of glucose from maltose or glycogen (Jeffrey et al, 1970). The enzyme and substrate were mixed in 0.1 M acetate buffer pH 4 or 5.5 depending on the enzyme, and incubated at 37°C in a shaker bath for the required time intervals. The reaction was quenched by placing the tube in a boiling water bath for 3 minutes and the glucose released was determined by the hexokinase method (Keller, 1965).

2.1.2 Analytical methods

a. Protein determination

Protein was determined by the method of Lowry et al (1951). To the protein sample in a final volume of water, add .5 ml of 1 N NaOH mix, and then add 5 ml of copper carbonate reagent (Sodium carbonate 4.0% 25 ml, distilled water 25 ml, $CuSO_4 \cdot 5H_2O$ 1.0% 1 ml, Na-K tartrate 2.5% 1 ml) and mix. Allow to stand for 10 minutes at room temperature and then add 0.5 ml of 1 N Folin reagent. Mix each tube by inversion immediately after adding the folin reagent. After 30 minutes read vs. a reagent blank at 620 nm.

b. Determination of glucose by the hexoquinase method

Glucose was determined by the hexoquinase method (Keller, 1965). One ml of Glucose (HK)20 reagent (Sigma Chemicals) was taken in a microcuvete, 100 µl of the sample was added and read at 340 nm for 5 minutes and the final stable reading was noted. Blank (one ml of Glucose (HK)20 reagent + 100 µl of the buffer) was subtracted and the reading was converted to µgs glucose using standard curve for glucose.

c. Determination of glucose content of glycogen

Glucose content of glycogen was determined by the method of Dubois et al (1956). Two milliliters of sugar solution containing between 10 and 70 µg of sugar is pipetted into a colorimetric tube and 0.05 ml of 80% phenol is added. Then 5 ml of concentrated sulfuric acid is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain a good mixing. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 minutes in a water bath at 25°C to 30°C, before readings are taken. The color is stable for several hours and readings may be made later if necessary. The absorbance

of the characteristic orange yellow color is measured at 490 nm. Blanks are prepared by substituting distilled water for the sugar solution. The amount of sugar may then be determined by reference to a standard curve previously constructed for the particular sugar under examination.

2.1.3 Preparation of enzyme-albumin polymers

Polymers of α-glucosidase and albumin were prepared according to the method of Poznansky (1983). Typical reaction mixture contains 2 mg glucosidase in 1 ml PBS (0.067 M potassium phosphate) pH 6.8, 20 mg bovine serum albumin in 1 ml PBS pH 6.8 and 6 mg PNPG (P-nitrophenyl α-D-glucopyranoside) for yeast and Aspergillus niger- α-glucosidase or 6 mg maltose for human placenta α-glucosidase in 600 µl of PBS pH 6.8 and 50 µl glutaraldehyde. Enzyme and maltose or PNPG were mixed and cooled to 4°C. To this albumin (cooled to 4°C) was added and stirred. Finally 50 µl of glutaraldehyde was added and allowed to react for 4 hours with continuous stirring. The mixture was then dialyzed against pH 6.8 overnight and finally dialyzed against PBS containing 1% glycine for 24 hours.

In order to prepare enzyme-albumin-insulin polymer, enzyme-albumin polymer was prepared as above except that the second dialysis was against 0.067 M sodium phosphate buffer pH 6.8. To this, 40 mg of insulin in 2 ml of 0.067 M phosphate buffer,

12 mg of PNPG and 50 µl of glutaraldehyde were added and stirred for 3 hours at 4 C and then dialyzed against PBS containing glycine for 24 hours.

2.1.4 Purification of human placenta α-glucosidase

Human placenta α-glucosidase was purified according to method of De Barsy et al (1972) with some modifications. Placentas were stored frozen at 20°C were thawed then homogenized in a Waring blender in 2 volumes of 1 mM EDTA-25 mM NaCl (standard buffer) (pH 5) and centrifuged at 8,000 rpm for 30 minutes. Supernatant was brought to pH 3.8 to 4 and centrifuged at 8,000 rpm for 30 minutes. The supernatant was collected and proteins were precipitated by adding 500 g ammonium sulfate per litre of supernatant. The preparation was left in the cold overnight then centrifuged at 10,000 rpm for 30 minutes. Precipitate was dissolved in the above buffer and dialyzed against the same buffer overnight. Sample was chromatographed in a Concanavalin A-Sepharose 4B column (.9 x 15 cm) eluted with mannose in standard buffer. Active fractions were pooled, concentrated, dialyzed and passed through a Sephadex G-100 (2.9 x 100 cm) column eluted with the same buffer containing 25% maltose, active fractions were pooled and concentrated.

2.2 MATERIALS

Yeast α-glucosidase, Aspergillus niger amyloglucosidase, bovine serum albumin Fraction V (98-99% albumin, remainder mostly globulins), PNPG, glutaraldehyde Grade I, insulin from bovine pancreas, maltose, glycogen from rabbit liver Type III, Glucose hexokinase Assay kit, Concanavalin A- Sepharose 4B, were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-100 was obtained from Pharmacia Fine Chemicals. Human placentas were obtained from University of Alberta Hospitals.

3. RESULTS

3.1 α-GLUCOSIDASE FROM YEAST

The activity of α-glucosidase from yeast and conjugates was investigated using maltose as substrate. The effects of enzyme concentration and period of incubation on the activity using maltose as substrate are shown in Fig. 3.1. The activity is linear for enzyme concentration up to 1.5 mg and for period of incubation up to 15 minutes. The effect of pH on activity is given in Fig. 3.2. The pH profiles indicate that both the monomer and albumin conjugate have a pH optimum of 6. The substrate saturation curves are given in Fig. 3.3. The Km for the monomer is 3.28 mM and for the albumin conjugate is 2.03 mM. The corresponding Lineweaver-Burk plots are shown in Fig. 3.4. The Km for the monomer is 3.22 mM and for albumin-conjugate is 2.09 mM. The Km and pH optimum are given in Table 3.1. Thermal stability of the enzyme incubated at 37°C is shown in Fig. 3.5. The monomer has 0% of initial activity by 24 hours while the albumin conjugate has 38% of initial activity by 24 hours.

FIGURE 3.1

Effect of enzyme concentration (A) and period of incubation (B) on yeast-α -glucosidase activity with maltose (0.138 M) as substrate in acetate buffer (0.075 M pH 5.5). For (A), a period of incubation of 5 minutes and for (B), an enzyme concentration of .25 mg/ml were used.

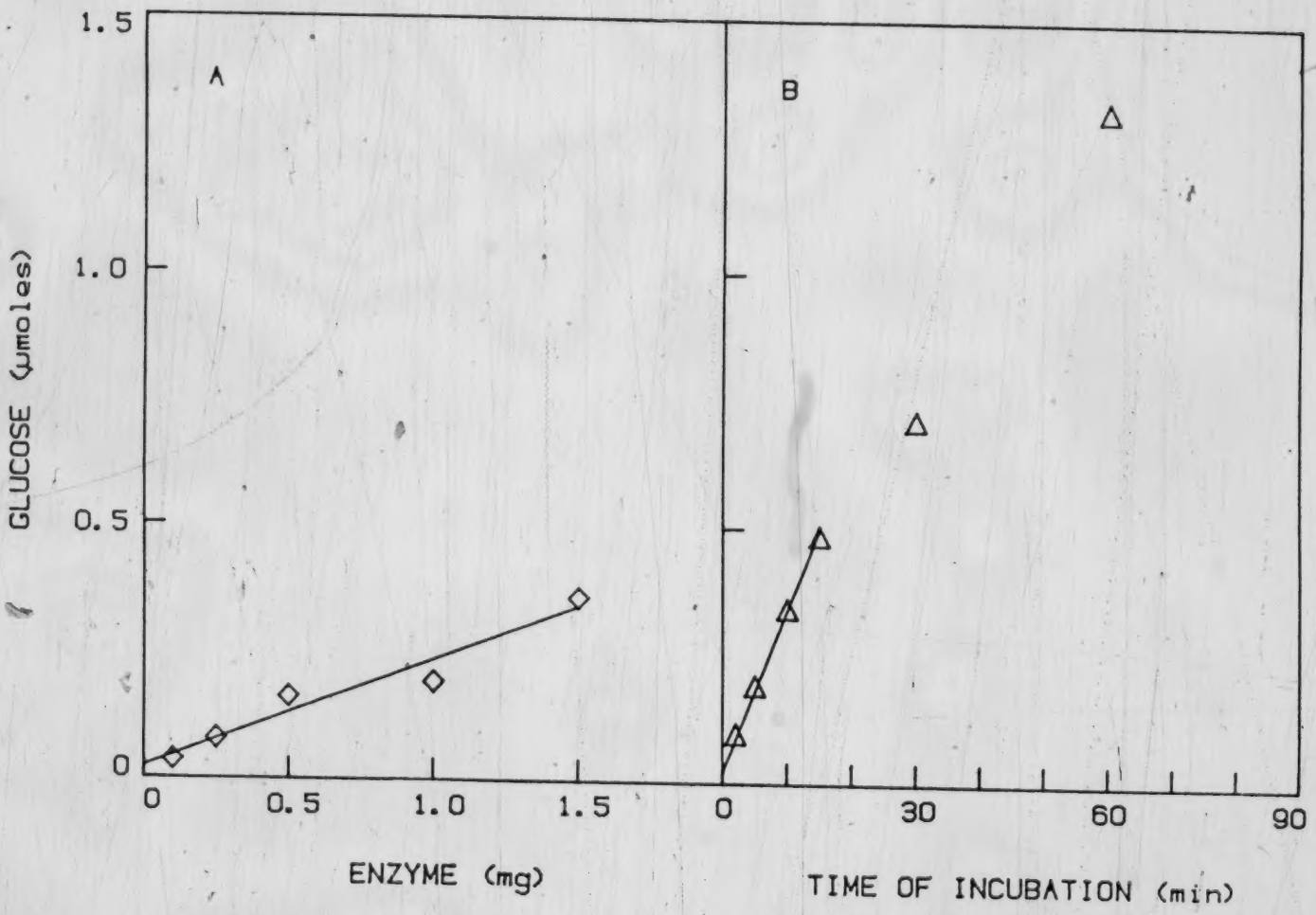
1.5
1.0
0.5
0
A
B
GLUCOSE (μmoles)
0
0.5
1.0
1.5
ENZYME (mg)
0
30
60
90
TIME OF INCUBATION (min)

FIGURE 3.2

pH-activity profiles of yeast-α-glucosidase (◇) and yeast-α-glucosidase-albumin conjugate (+) using maltose (0.046 M) as substrate in acetate buffer (0.75 M, pH 3.5 to 5.5) or phosphate buffer (0.055 M, pH 6 to 8) incubated at 37°C for 10 minutes, with a .25 and 1.34 mgs protein/ml respectively.

μmoles GLUCOSE/min/mg PROT. (◇ ×10 -3)
(+=×10 -4)
200
150
100
50
0
3
4
5
6
7
8
9
pH

FIGURE 3.3

Substrate saturation plots of yeast-α-glucosidase (◇) and yeast-α-glucosidase-albumin polymer (+) using maltose as substrate in acetate buffer (0.075 M, pH 5.5) incubated 37°C for 10 minutes with .25 and 1.34 mg protein/ml respectively.

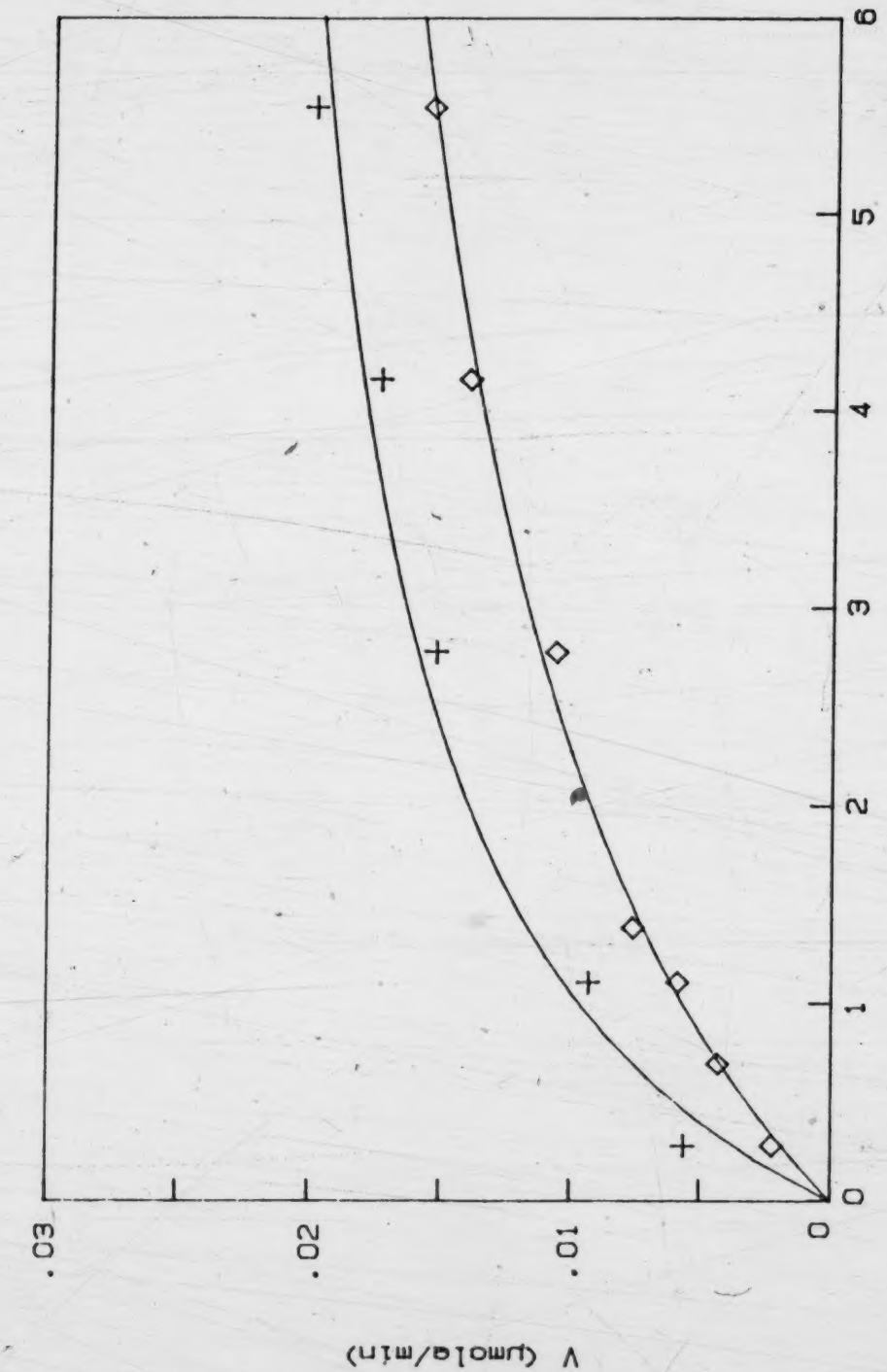
V (μmole/min)
.03
.02
.01
0
0
1
2
3
4
5
6
S (mM)

FIGURE 3.4

Lineweaver-Burk plots for the data given in Fig. 3.3. Yeast- α-glucosidase (◇) and yeast- α-glucosidase-albumin polymer (+).

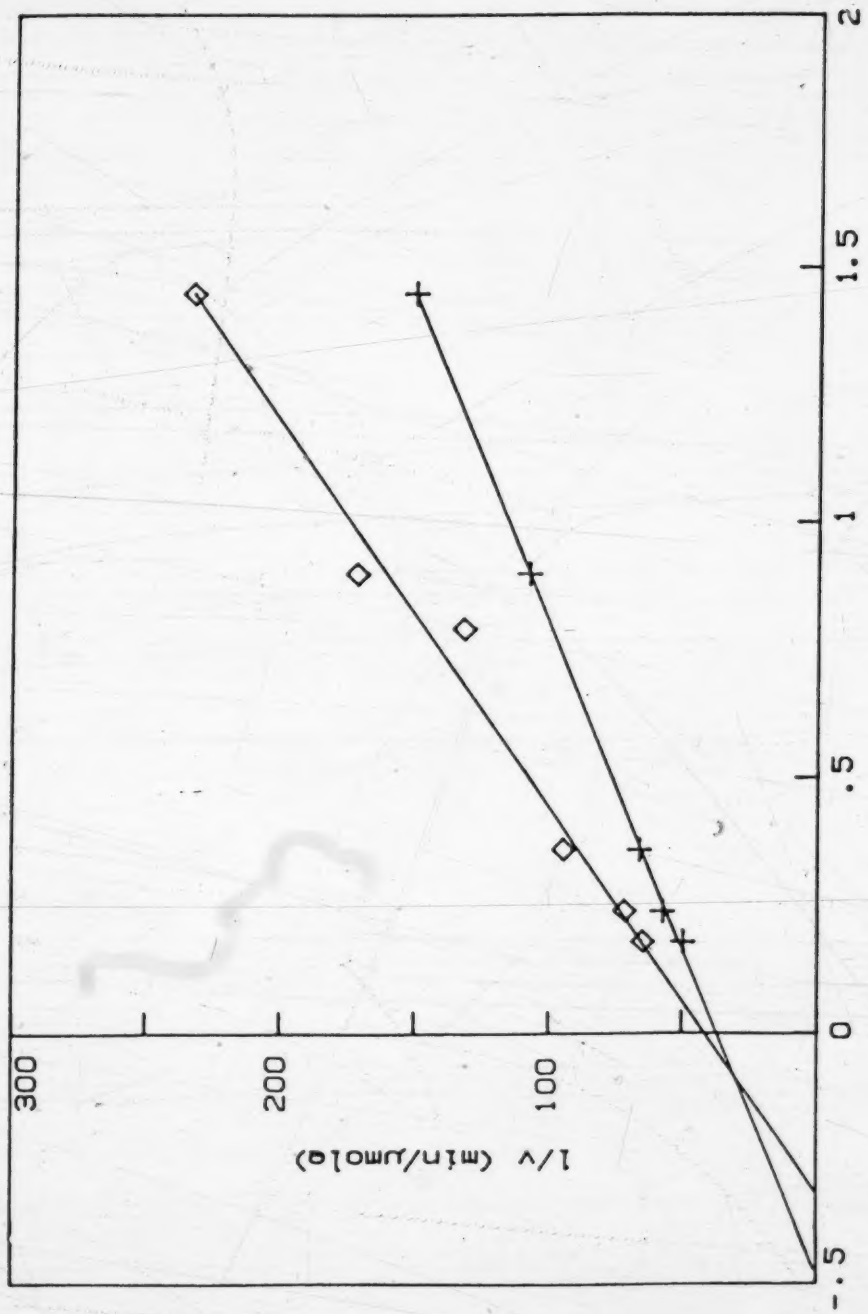
1/v (min/μmole)
300
200
100
0
-.5
.5
1
1.5
2
1/S (mM^{-1})

FIGURE 3.5

Thermal stability at 37°C of yeast-α-glucosidase (◇) and yeast-α-glucosidase-albumin polymer (+) using maltose (0.046 M) as substrate in acetate buffer (0.083 M, pH 5.5), .25 mg/ml of monomer and 1.34 mg/ml of polymer were incubated at 37°C and 100 µl. Aliquots were withdrawn at different time intervals and assayed for α-glucosidase activity at 37°C as usual.

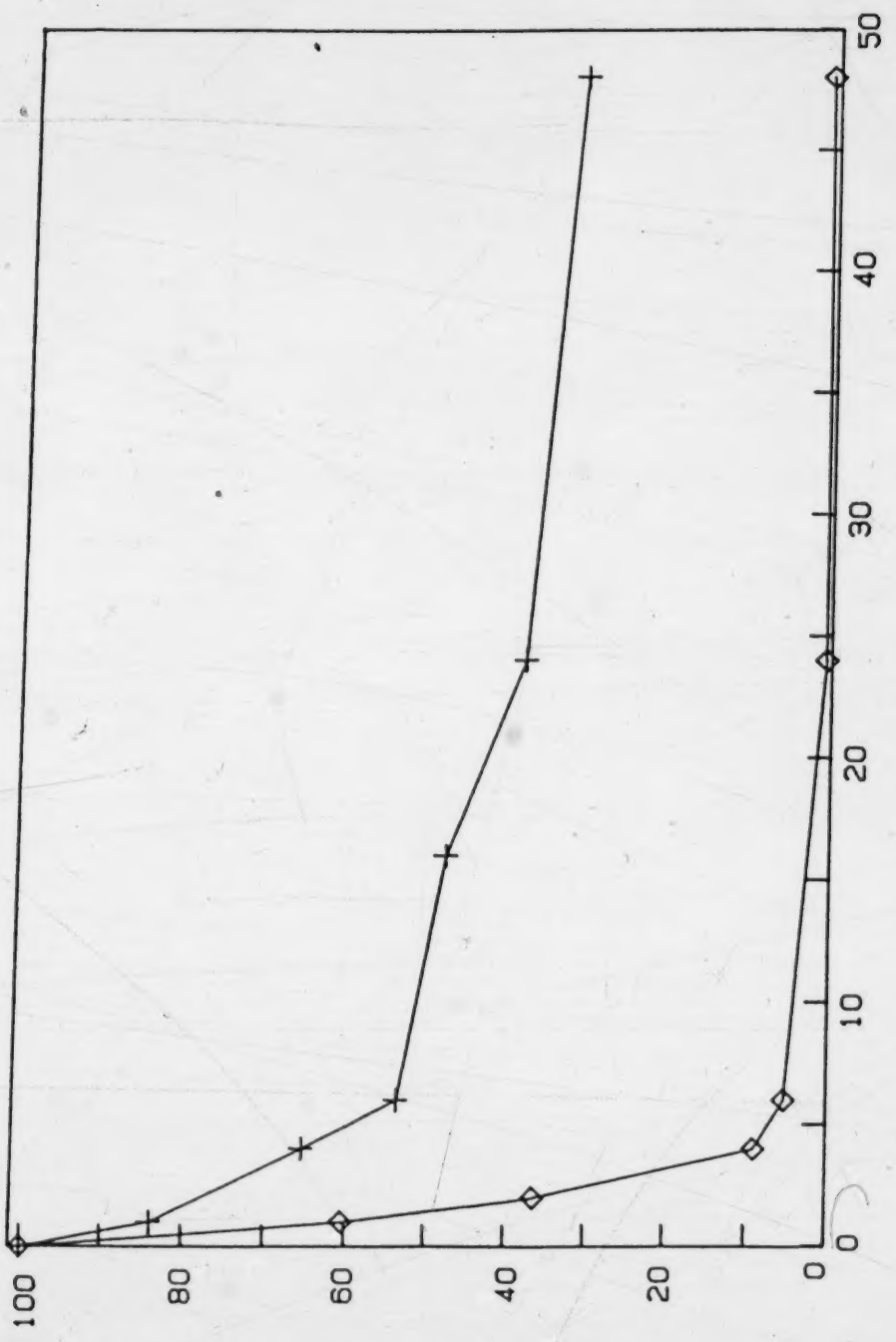
RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
TIME (hrs)

TABLE 3.1

Km and pH Optima for Different forms of yeast-α-glucosidase

Form of Enzyme	Substrate	Km(mM)	pH Optimum
α-glucosidase monomer *	Maltose	3.22	6
α-glucosidase-albumin polymer (pH 6.8) *	Maltose	2.09	6

* Monomer means the native form of the enzyme. Polymer means enzyme conjugated to some other protein, the polymer was prepared at pH 6.8.

3.2 α-GLUCOSIDASE FROM ASPERGILLUS NIGER

The activity of α-glucosidase from A. niger and conjugates was investigated using maltose and glycogen as substrates. The effects of enzyme concentration and period of incubation on the activity using maltose as substrate are shown in Fig. 3.6. The activity is linear for enzyme concentration up to 0.06 mg and for a period of incubation (minimum) up to 60 minutes. The effect of pH on activity is given in Fig. 3.7. The pH profiles indicate that the monomer has a pH optimum of 4 while albumin conjugate (pH 6.8) has a value of 4.5, albumin conjugate (pH 4.5) has a value of 3 and albumin-insulin conjugate has a value of 3.5. The substrate saturation curves are given in Fig. 3.8. The Km for the monomer is 0.113 mM, while albumin conjugate (pH 6.8) has a value of 0.14 mM, albumin conjugate (pH 4.5) has a value of 0.135 mM and albumin insulin conjugate has a value of 0.14 mM. The corresponding Lineweaver-Burk plots are shown in Fig. 3.9. The Km for the monomer is 0.108 mM, while albumin conjugate (pH 6.8) has a value of 0.147 mM, albumin conjugate (pH 4.5) has a value of 0.13 mM and albumin-insulin conjugate has a value of 0.142 mM. The Km and pH optimum values are shown in Table 3.2. Thermal stability is shown in Fig. 3.10. The monomer and albumin conjugate (pH 6.8) have about 100% of initial activity by 48 hours while the albumin-insulin conjugate has 27% of the initial activity by 48 hours.

The effects of enzyme concentration and the period of incubation on the activity using glycogen as substrate are shown in Fig. 3.11. The activity is linear for enzyme concentration up to 0.02 mg and for a period of incubation up to 30 minutes. The effect of pH on activity is given in Fig. 3.12. The pH profiles indicate that the monomer has a pH

optimum of 5, while albumin conjugated at pH 6.8 has a value of 3.5, albumin conjugated at pH 4.5 has a value of 4.5 and albumin-insulin conjugate has a value of 4. The Lineweaver-Burk plots are shown in Fig. 3.13. The Km for the monomer is 0.0109 mM, for albumin conjugate (pH 6.8) is 0.0067 mM, for albumin conjugate (pH 4.5) is 0.032 mM and that albumin-insulin conjugate is 0.006 mM. The Km and pH optimums are shown in Table 3.2. The thermal stability of the enzyme preparations at 37°C assayed using glycogen is shown in Fig. 3.14. The monomer and albumin conjugate (pH 6.8) have about 100% of initial activity by 48 hours.

FIGURE 3.6

Effect of enzyme concentration (A) and period of incubation (B) on Aspergillus niger- α -glucosidase activity with maltose (0.070 M) in acetate buffer (0.075 M, pH 4.5). For (A), a period of incubation of 20 minutes and for (B), an enzyme concentration of .01 mg/ml were used.

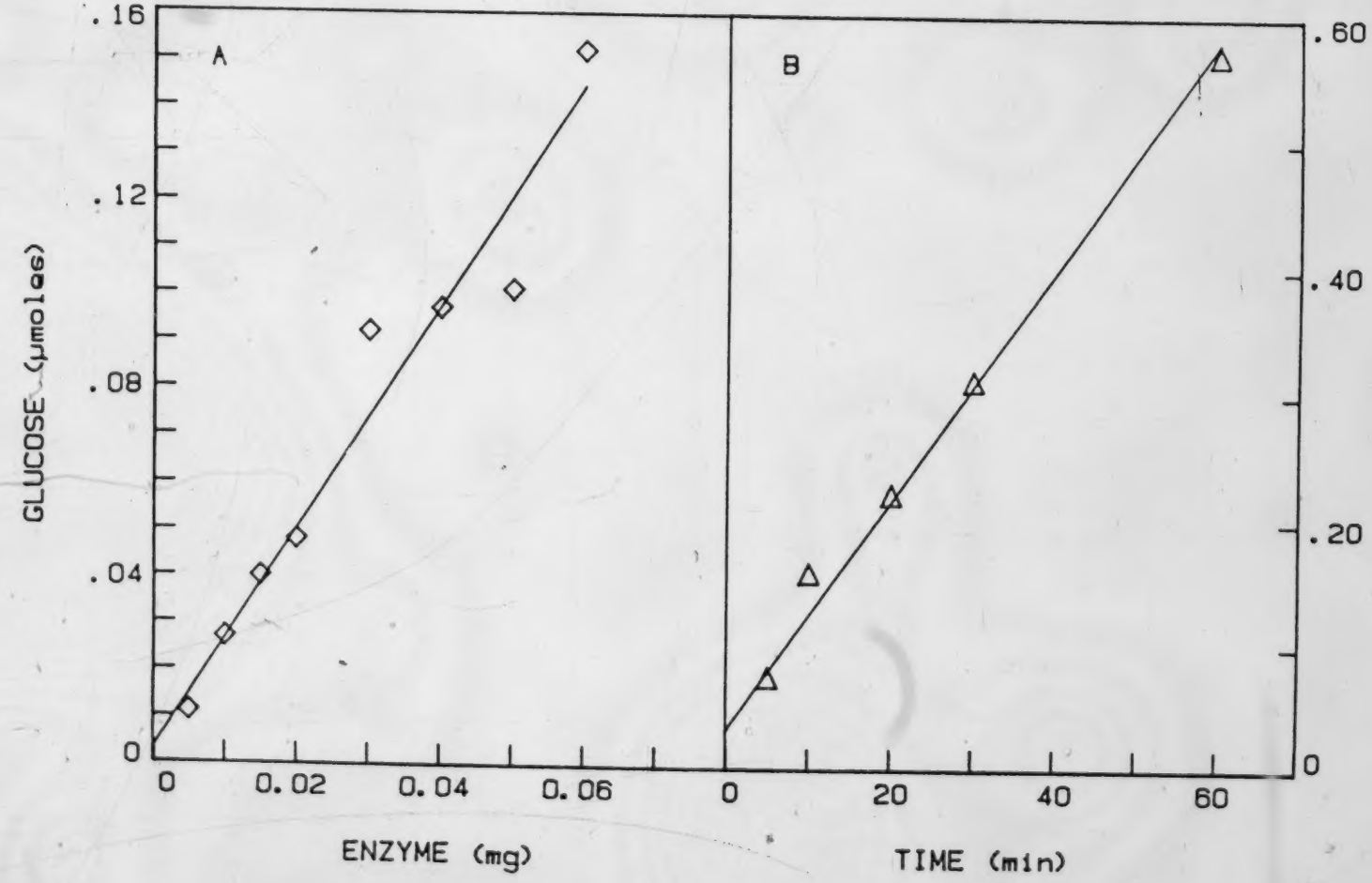
A
B
.16
.12
.08
.04
0
.60
.40
.20
0
0
0.02
0.04
0.06
0
20
40
60
GLUCOSE (µmoles)
ENZYME (mg)
TIME (min)

FIGURE 3.7

pH-activity profiles for α-glucosidase from A. niger. Monomer (◇), α-glucosidase-albumin polymer (pH 6.8) (+), α-glucosidase-albumin polymer (pH 4.5) (▽) and α-glucosidase-albumin-insulin polymer (▲) using maltose (0.0031 M) as substrate in glycine buffer (0.083 M, pH 2.5 to 3), acetate buffer (0.083 M, pH 3.5 to 5.5) and phosphate buffer (0.056 M, pH 6 to 8) and incubated at 37°C for 10 minutes with .01, .220, .220 and .234 mg protein/ml respectively.

μmoles glucose /mg prot.
(◇)
μmoles glucose/mg prot.
(+, △, ▲ = x 10 -2)
pH
0
2
4
6
8
10
12
0
20
40
60
80
100
120
2
3
4
5
6
7
8
9

FIGURE 3.8

Substrate saturation plots of Aspergillus niger- α -glucosidase. Monomer (◇), α -glucosidase-albumin polymer (pH 6.8)(+), α -glucosidase-albumin polymer (pH 4.5)(△) and α -glucosidase-albumin-insulin polymer(▽) using maltose as substrate in acetate buffer (0.083 M, pH 4.5) incubated at 37°C for 10 minutes with .01, .220, .220 and .234 mgs protein/ml respectively.

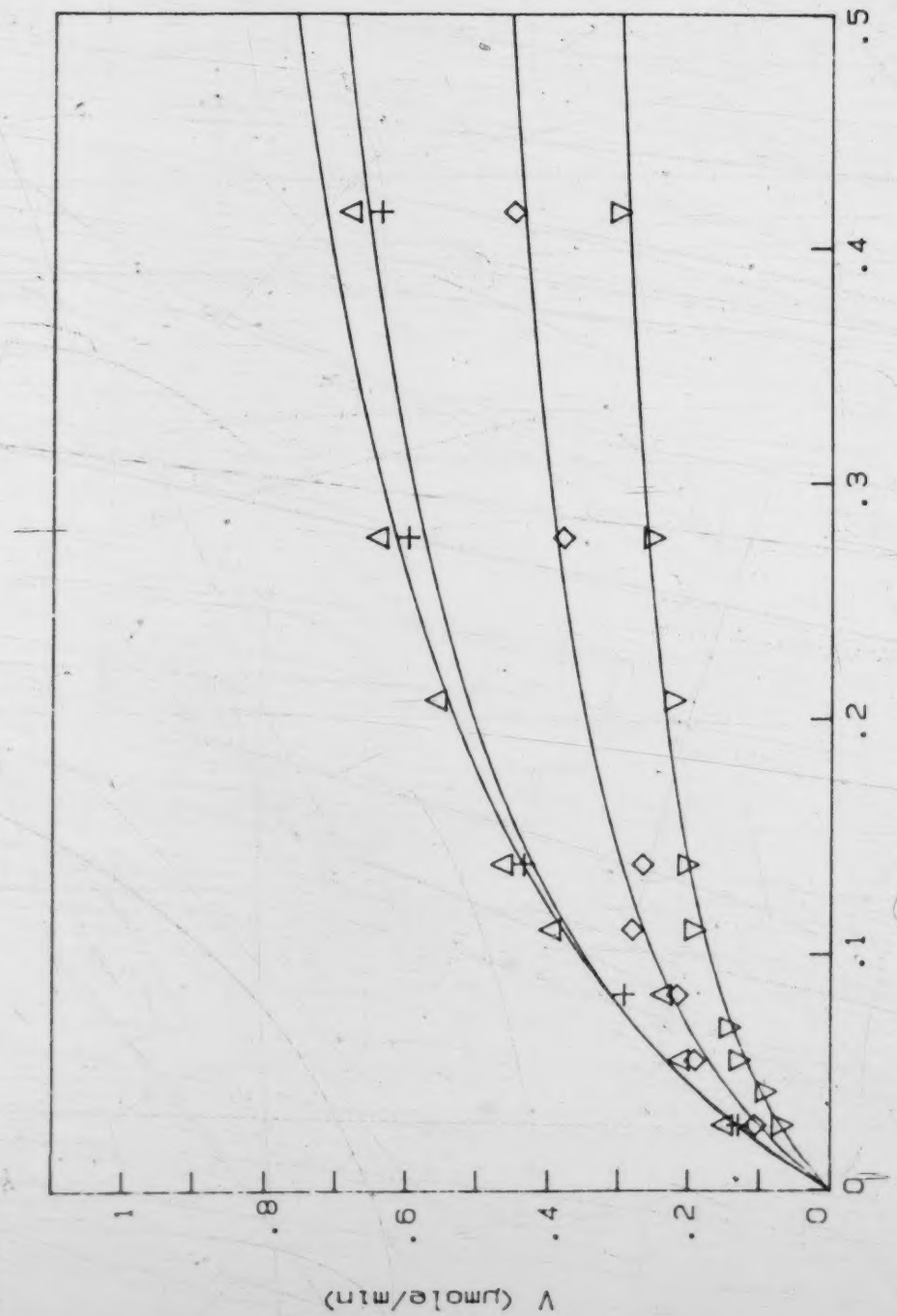
V (μmole/min)
0
.2
.4
.6
.8
1
0
.1
.2
.3
.4
.5
S (mM)

FIGURE 3.9

Lineweaver-Burk plots for the data given in Fig. 3.8. A. niger- α-glucosidase (◇), α-glucosidase-albumin (pH 6.8)(+), α-glucosidase-albumin polymer (pH 4.5)(▽) and α-glucosidase-albumin-insulin polymer (△).

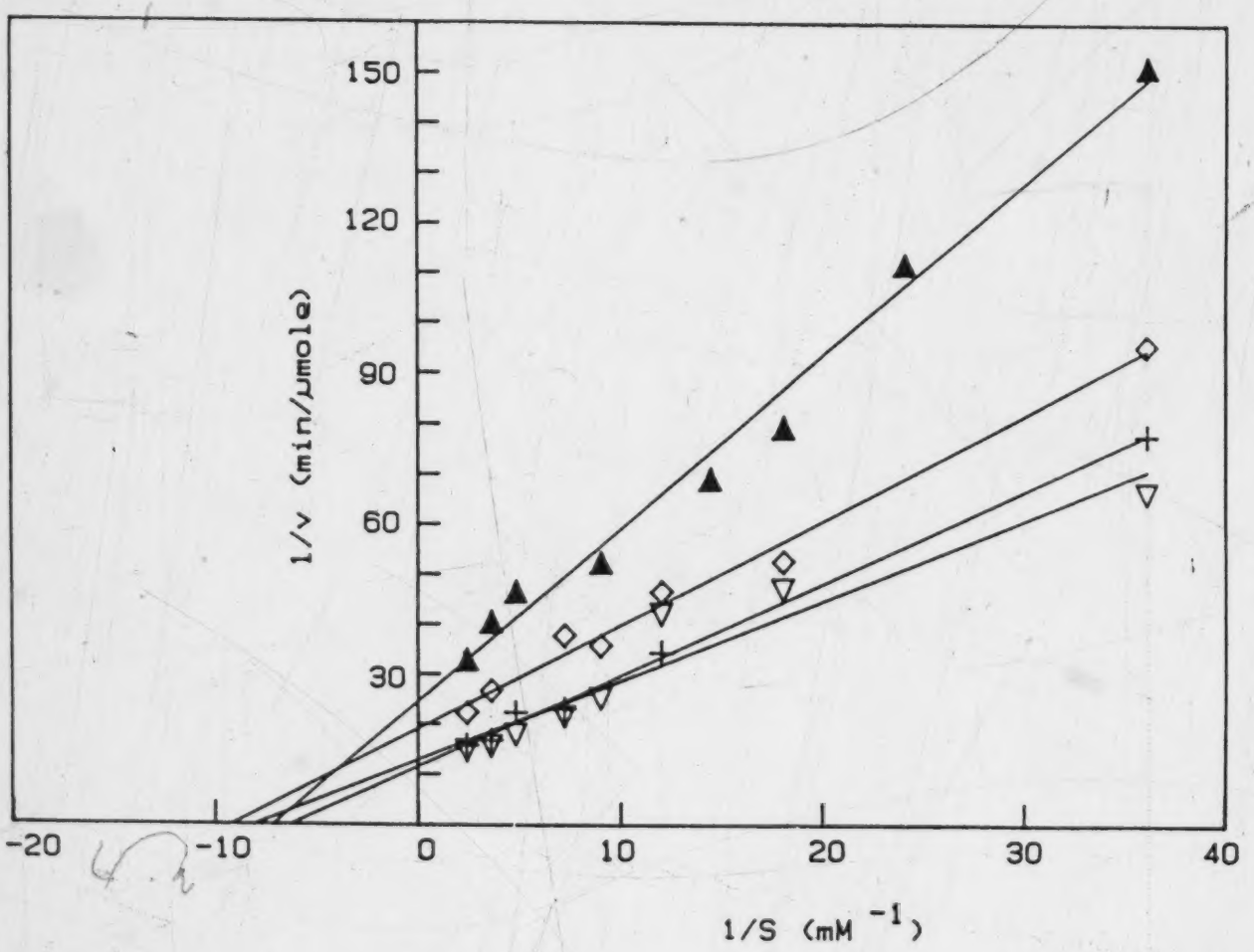
150
120
90
60
30
1/v (min/μmole)
-20
-10
0
10
20
30
40
1/S (mM $^{-1}$)

FIGURE 3.10

Thermal stability at 37°C of Aspergillus niger- α-glucosidase (◇), Aspergillus niger-albumin (pH 6.8)(+) and Aspergillus niger-albumin-insulin (△) using maltose (0.0055 M) as substrate in acetate buffer (0.083 M, pH 4.5). .01 mg/ml of monomer, .220 mg/ml of albumin polymer (pH 6.8) and .234 mg/ml of albumin insulin polymer were incubated 37°C and 100 µl aliquots were withdrawn at different time intervals and assayed for α-glucosidase activity at 37°C as usual.

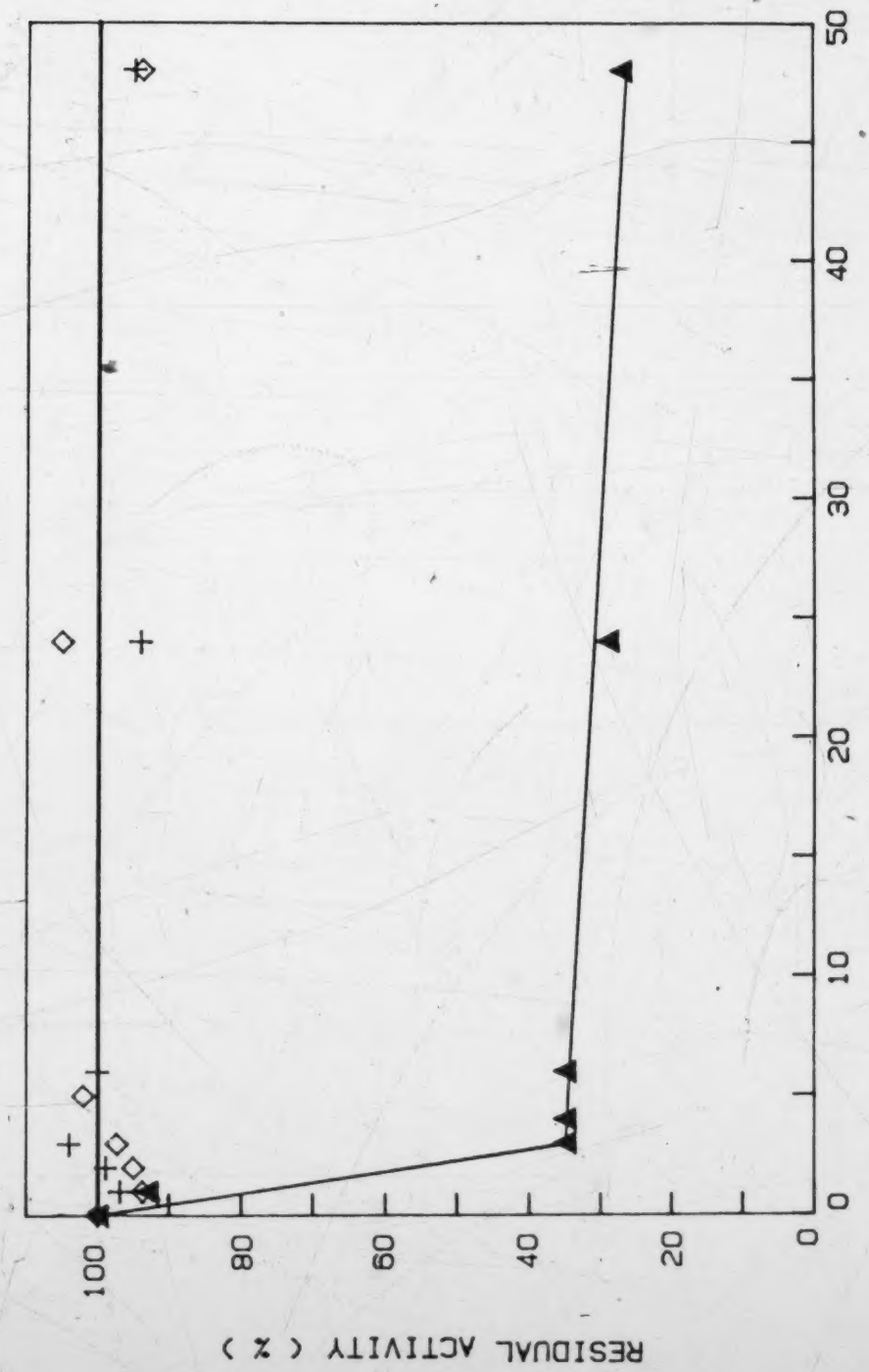
RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
TIME (hours)

FIGURE 3.11

Effect of enzyme concentration (A) and period of incubation (B) on Aspergillus niger- α -glucosidase activity with glycogen 0.25% as substrate in acetate buffer (0.075 M). The enzyme stock stolution contained .2 mg/ml. For (A), a period of incubation of 20 minutes and for (B), an enzyme concentration of .01 mg/ml were used.

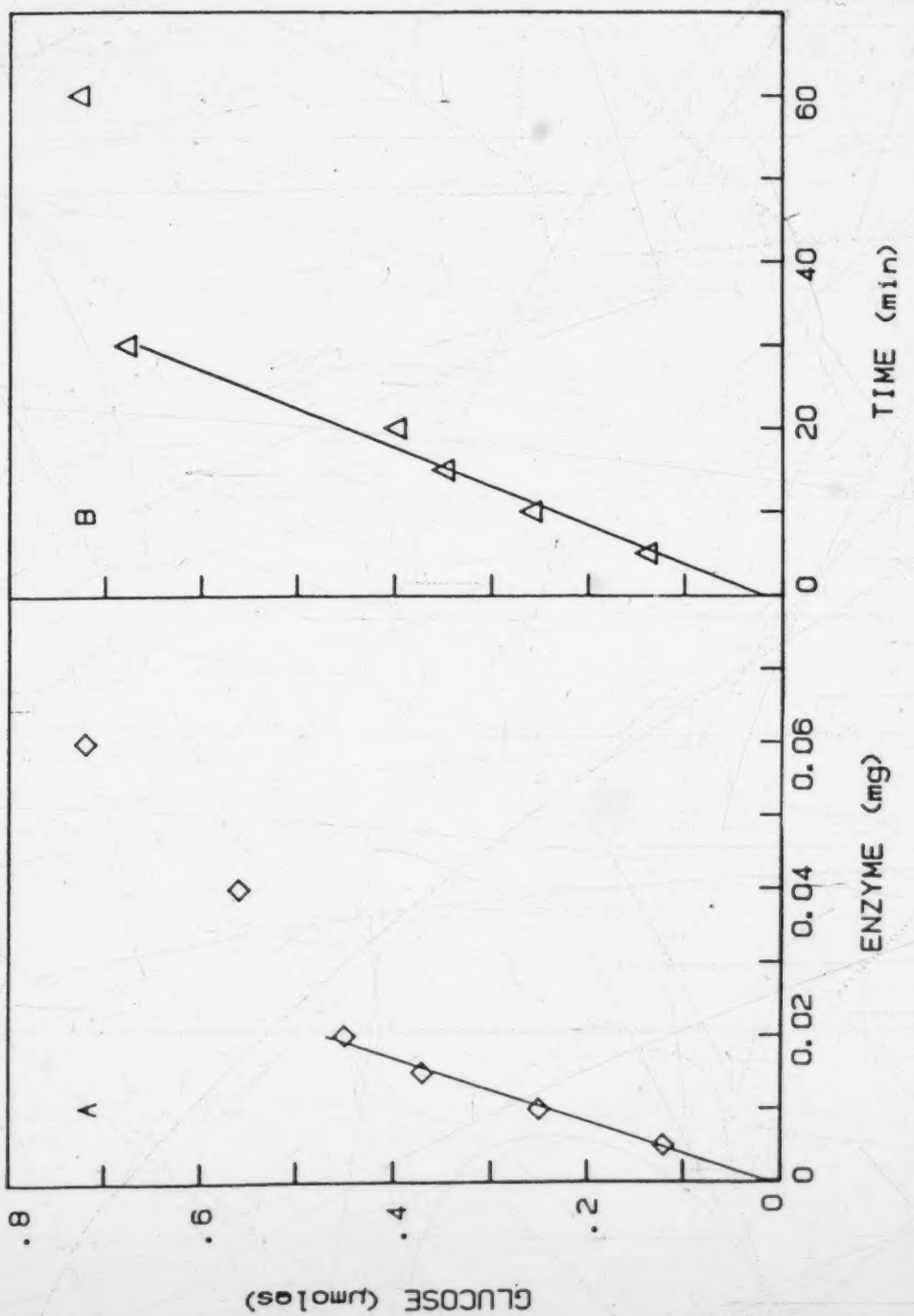
GLUCOSE (μmoles)
.8
.6
.4
.2
0
A
0
0.02
0.04
0.06
ENZYME (mg)
B
0
20
40
60
TIME (min)

FIGURE 3.12

pH-activity profiles for α-glucosidase from Aspergillus niger. Monomer (◇), α-glucosidase-albumin polymer (pH 6.8)(+) and (B) α-glucosidase-albumin (pH 4.5)(◇) and α-glucosidase-albumin-insulin polymer(+) using glycogen 0.11% as substrate in glycine buffer (0.083 M, pH 2.5 to 3), acetate buffer (0.083 M, pH 3.5 to 5.5) and phosphate buffer (0.056 M, pH 6 to 8) incubated at 37°C for 15 minutes with .0084, .099, .099 and .234 mgs protein/ml respectively.

A
B
pH
umolae glucose/mg prot. (◇)
umolae glucose/mg prot. (+ = × 10⁻²)
umolae glucose/mg prot. (+ × 10⁻²)

FIGURE 3.13

Lineweaver-Burk plots of Aspergillus niger- α-glucosidase. Monomer (◇), α-glucosidase-albumin polymer (pH 6.8)(+) and α-glucosidase-albumin-insulin polymer (▼) using glycogen as substrate in acetate buffer (0.083 M, pH 4.5) incubated at 37°C for 15 minutes with .0084, .099, .099 and .234 mgs protein/ml respectively.

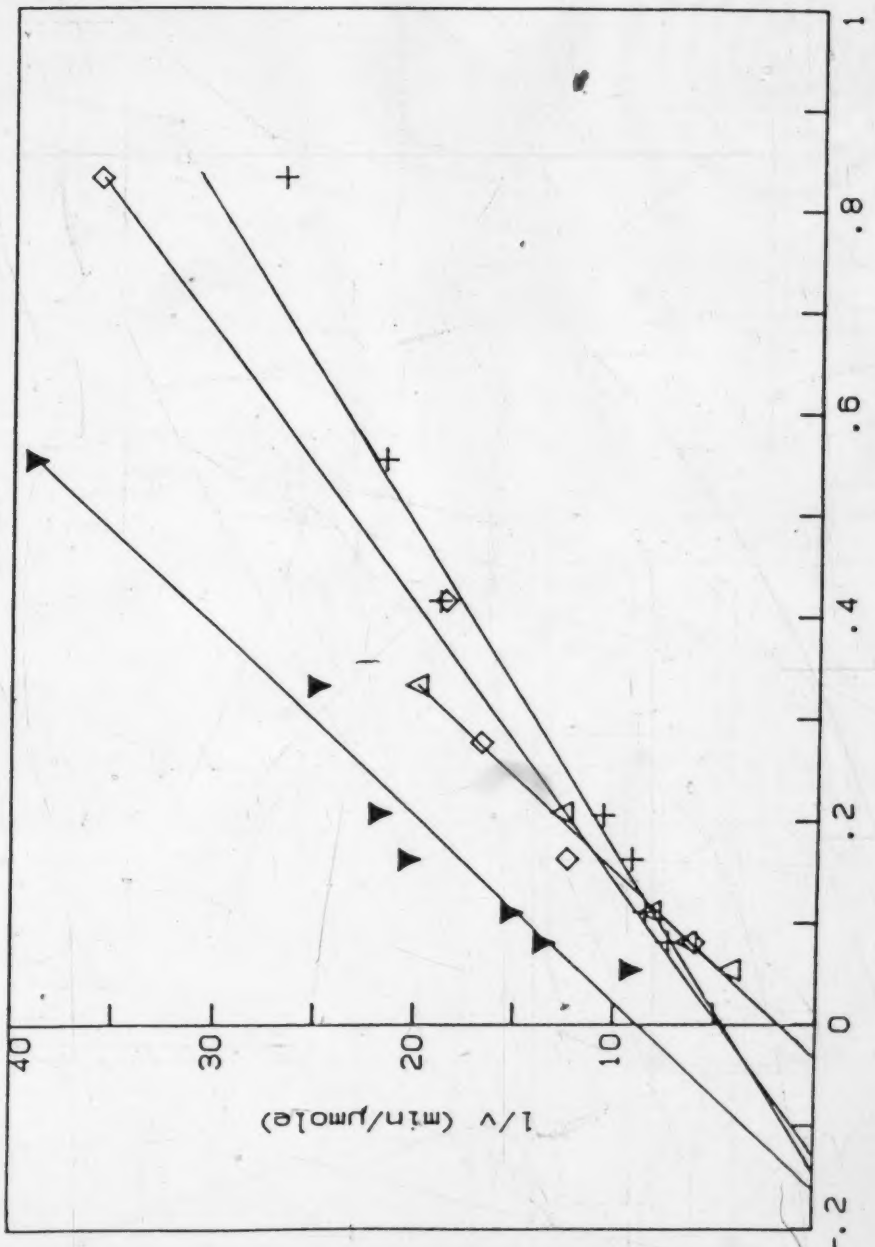
1/v (min/μmole)
40
30
20
10
0
-.2
.2
.4
.6
.8
1
1/S (μM⁻¹)

FIGURE 3.14

Thermal stability at 37°C of Aspergillus niger- α-glucosidase (◇) and A. niger- α-glucosidase-albumin (pH 6.8)(+) using glycogen 0.20% as substrate in acetate buffer (0.083 M, pH 4.5). .0084 and .099 mg protein/ml respectively were incubated 37°C and 100 μl aliquots were withdrawn at different time intervals and assayed for α-glucosidase activity at 37°C as usual.

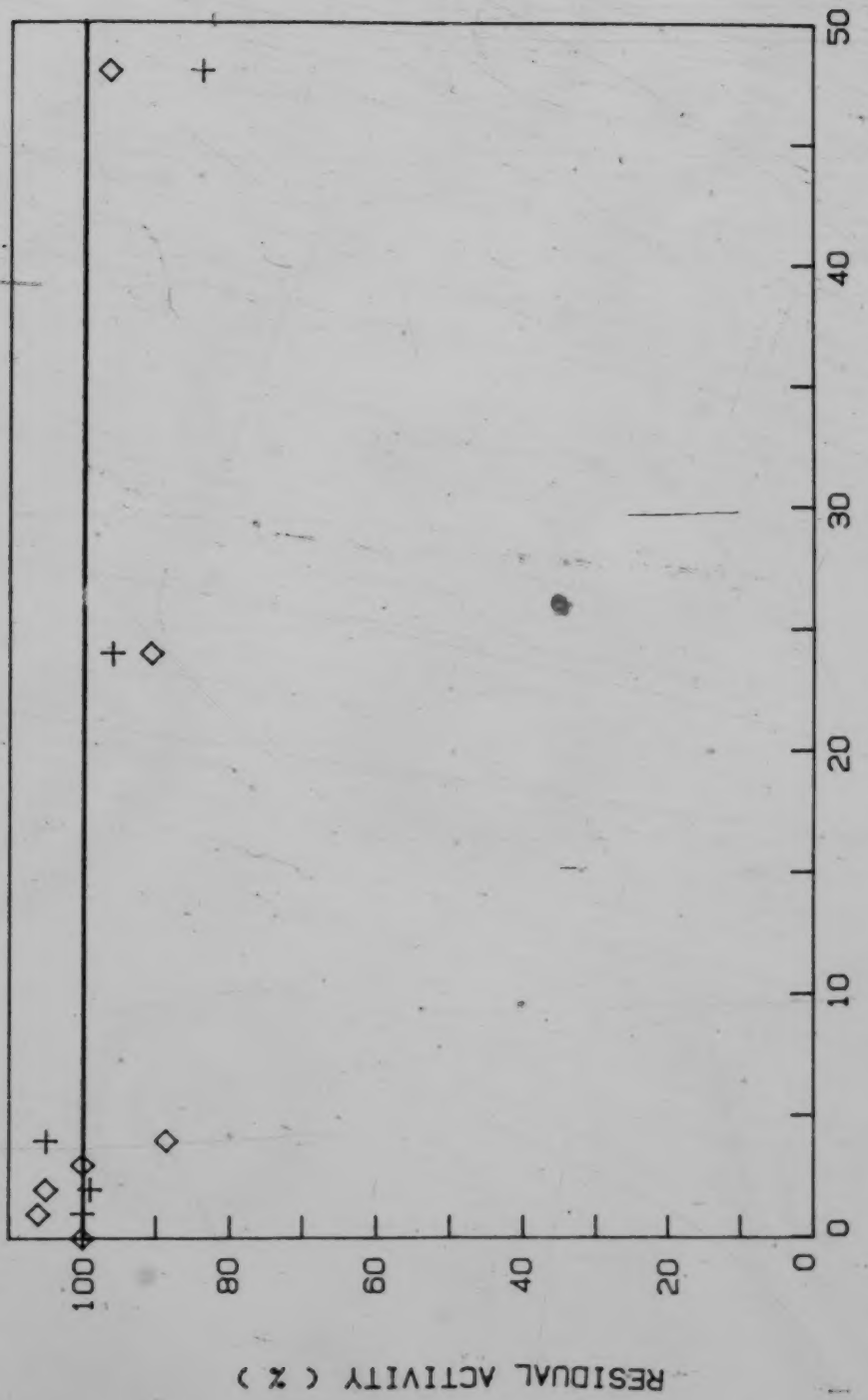
RESIDUAL ACTIVITY (%)
100
80
60
40
20
0
0
10
20
30
40
50
TIME (hours)

TABLE 3.2

Km and pH optima for different forms of
Aspergillus niger- α -glucosidase

Form of Enzyme	Substrate	Km(mM)	pH optimum
α -glucosidase monomer *	Maltose	.108	4.0
α -glucosidase-albumin (pH 6.8) polymer *	Maltose	.147	4.5
α -glucosidase-albumin (pH 4.5) polymer *	Maltose	.13	3.0
α -glucosidase-albumin-insulin polymer	Maltose	.142	3.5
α -glucosidase monomer	Glycogen	.0109	5.0
α -glucosidase-albumin (pH 6.8) polymer	Glycogen	.0067	3.5
α -glucosidase-albumin (pH 4.5) polymer	Glycogen	.032	4.5
α-glucosidase-albumin-insulin polymer	Glycogen	.006	4.0

* Monomer means the native form of the enzyme. Polymer means enzyme conjugated to some other protein. The polymers were prepared in buffers of indicated pH values.

3.3 α-GLUCOSIDASE FROM HUMAN PLACENTA

The activity of α-glucosidase from human placenta and conjugates was investigated using maltose and glycogen as substrates. The effect of pH on activity using maltose as substrate is given in Fig. 3.15. The pH profiles indicate that the monomer has a pH optimum of 4 and albumin conjugate (pH 6.8) has a value of 4, while albumin conjugate (pH 4.5) has a value of 3 and albumin-insulin conjugate has a value of 4.5. The Lineweaver-Burk plots are shown in Fig. 3.16. These plots gave Km values of .44, .66, .66 and .77 mM for monomer, albumin conjugate (pH 6.8), albumin conjugate (pH 4.5) and albumin-insulin conjugate respectively. The effect of pH on activity using glycogen as substrate is given on Fig. 3.17. The pH profiles indicate that the monomer has a pH optimum of 3.5 while the albumin conjugate (pH 6.8) has 3. The Lineweaver-Burk plots are shown in Fig. 3.18. The Km for the monomer is 0.39 mM, for albumin-conjugate (pH 6.8) is 0.065 mM and for albumin conjugate (pH 4.5) is 0.083 mM. The Km and pH optimum values are shown in Table 3.3.

FIGURE 3.15

pH-activity profiles for α-glucosidase from human placenta. Monomer (◇), α -glucosidase-albumin polymer (pH 6.8)(+), α-glucosidase-albumin polymer (pH 4.5)(△) and α-glucosidase-albumin-insulin (▼) using maltose (0.0138 M) as substrate in glycine buffer (0.05 M, pH 2.5 to 3), acetate buffer (0.05 M, pH 3.5 to 5.5) and phospate buffer (.033 M, pH 6 to 8) incubated at 37°C for 30 minutes with .0036, .207, .207 and .530 mg protein/ml respectively.

μmolae glucose/mg prot. (+,▽ = x 10⁻²)
μmolae glucose/mg prot. (◇ = x 10⁻¹)
(▲ = x 10⁻²)
300
200
100
0
100
75
50
25
0
2
3
4
5
6
7
8
9
pH

FIGURE 3.16

Lineweaver-Burk plots of human placenta-α-glucosidase. (A) Monomer (◇) and (B)-α-glucosidase-albumin polymer (pH 6.8)(+), α-glucosidase-albumin polymer (pH 4.5)(△) and α-glucosidase-albumin-insulin polymer (▼) using maltose as substrate in acetate buffer (0.05M, pH 4) incubated at 37°C for 30 minutes with .0036, .207, .207 and .530 mg protein/ml respectively.

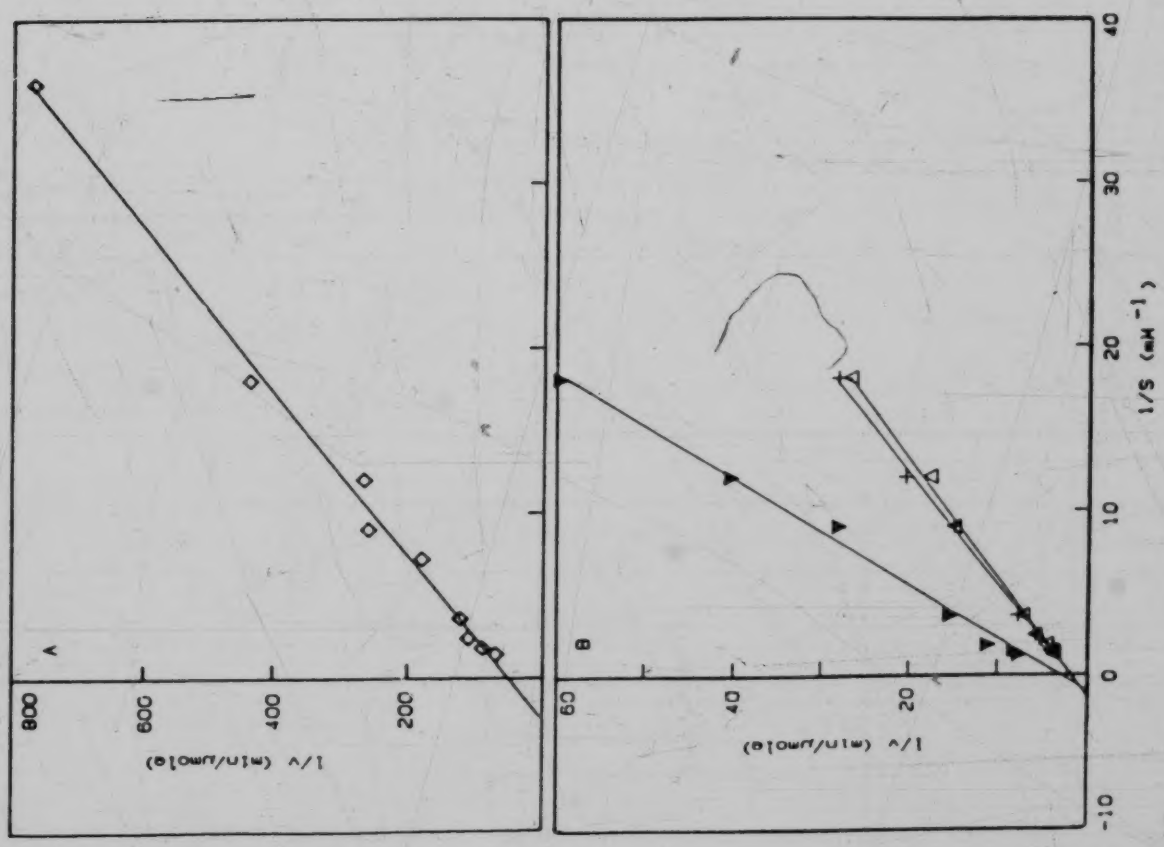
A
B
1/v (min/μmole)
800
600
400
200
60
40
20
0
10
20
30
40
-10
1/S (mM⁻¹)

FIGURE 3.17

pH-activity profiles for α-glucosidase from human placenta. Monomer (◇) and α-glucosidase-albumin polymer (+)(pH 6.8) using glycogen 1% as substrate in glycine buffer (0.05 M, pH 2.5 to 3), acetate buffer (0.05M, pH 3.5 to 5.5) and phosphate buffer (pH 6 to 8) incubated at 37°C for 60 minutes with .0018 and .414 mg protein/ml respectively.

μmolae glucoge/min/mg prot.
(◇ = x 10 -2 . + = x 10 -4)
200
150
100
50
0
2
3
4
5
6
7
8
9
pH

FIGURE 3.18

Lineweaver-Burk plots of human placenta- α -glucosidase. Monomer (◇), α-glucosidase-albumin polymer (pH 6.8)(+) and α-glucosidase-albumin polymer (pH 4.5)(△) using glycogen (1%) as substrate in acetate buffer (0.05 M, pH 4) incubated at 37°C for 60 minutes with .0018, .414 and .414 mg protein/ml respectively.

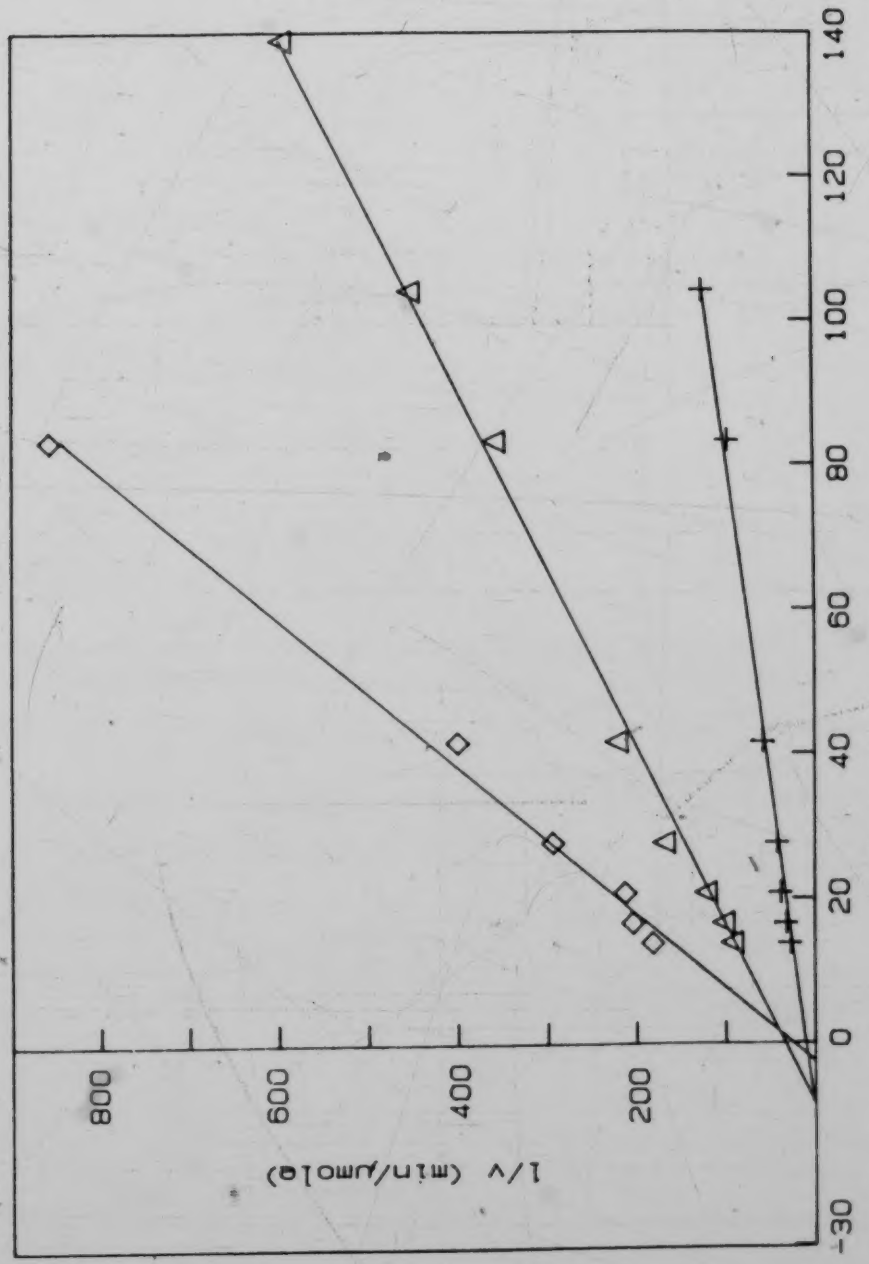
1/v (min/μmole)
800
600
400
200
0
-30
20
40
60
80
100
120
140
1/S (mM^{-1})

TABLE 3.3

Km and pH optima for different forms of human placenta- α -glucosidase

Form of Enzyme	Substrate	Km (mM)	pH Optimum
α -glucosidase monomer	Maltose	.44	4.0
α -glucosidase-albumin (pH 6.8) polymer	Maltose	.66	4.0
α -glucosidase-albumin (pH 4.5) polymer	Maltose	.66	3.0
α -glucosidase-albumin-insulin	Maltose	.77	4.5
H.P.- α-glucosidase	Glycogen	.39	3.5
H.P.- α-glucosidase-albumin (pH 6.8)	Glycogen	.065	3.0
H.P.- α -glucosidase-albumin (pH 4.5)	Glycogen	.083	---

4. DISCUSSION

α-glucosidases from 3 different sources were conjugated with a 10 molar excess albumin using glutaraldehyde as the cross-linking agent. The resultant polymers were characterized for pH sensitivity, Km and thermal stability using maltose and glycogen as substrate. The α-glucosidase-albumin polymers were further conjugated to insulin using glutaraldehyde as a cross-linking agent and characterized for pH sensitivity, Km and thermal stability. The pH optimum for the yeast-α-glucosidase monomer using maltose as substrate was 6 with a sharp curve. The pH optimum for the albumin conjugate was also 6 and the curve was broader than that for the monomer, which could mean that conjugation with albumin renders the enzyme more stable to changes in pH, probably due to changes in conformation in the active site. Stabilization of enzyme activity after conjugation with albumin has been reported with uricase-albumin conjugates (Poznansky, 1977). The Km for the yeast- α-glucosidase monomer using maltose as a substrate was 3.22 mM while that of the albumin conjugate was 2.09 mM indicating that the conjugate has more affinity for the substrate than the monomeric enzyme. At 37°C, yeast- α-glucosidase looses the activity completely by 24 hours whereas the albumin conjugate has a 38% of initial activity by 24 hours. This increase in thermal stability for enzyme-albumin conjugates has been previously reported for Uricase and yeast- α-glucosidase (Paillot et al, 1974, Poznansky, 1977, Poznansky and Bhardwaj, 1980). The yeast enzyme is not active towards glycogen. The pH optimum for the A. niger monomer using maltose as substrate was 4, for albumin conjugate (pH 6.8) 4.5, for albumin conjugate (pH 4.5) 3 and for albumin-insulin conjugate 3.5. The curves for the three

conjugates have a broader shape compared to that of the monomeric form which could mean, as stated earlier, that the enzyme is more stable because of the conjugation with albumin due to conformational changes in the active site. There was an acidic shift for albumin conjugate (pH 4.5) and albumin-insulin conjugate. This shift to more acid pH will not affect the performance of the conjugates at the lysosomal pH optimum near 5 because the enzyme is more stable in a wider range of pH. The Km for the A. niger monomer using maltose as substrate is 0.108 mM, for albumin conjugate (pH 4.5) is 0.13 mM, for albumin conjugate (pH 6.8) is .147 and for albumin-insulin conjugate is 0.142 mM indicating a slight decrease in affinity of the conjugated forms. At 37°C the monomeric and the albumin conjugate (pH 6.8) forms were stable for up to 48 hours. While the insulin conjugate has only 27% of the initial activity. This indicates that the monomeric form is stable, conjugation with albumin does not alter its stability and that further conjugation with insulin decreases its stability probably due to conformational changes in the active site of the enzyme during conjugation with insulin.

The pH optimum for A. niger monomer using glycogen as substrate is 5, for the albumin conjugate (pH 6.8) is 3.5 and for albumin conjugate (pH 4.5) is 4.5. The curves of both albumin conjugates are broader indicating that the enzyme is more stable at a wider pH range probably due to conformational changes in the active site. The shift to more acidic pH does not affect the overall performance of the conjugates at lysosomal optimum pH near 5 because of the broadening of the curves and a more stable enzyme at a wider range of pH. The pH optimum for the albumin-insulin conjugate is 4 with a small broadening of the shape of

the curve with the same implications stated above. The Km for the monomer, albumin-conjugate (pH 6.8 and 4.5) and albumin-insulin conjugate are 0.0109, 0.0067, 0.032 and 0.006 mM respectively indicating an increase of affinity for the substrate for albumin conjugate (pH 6.8) and albumin-insulin conjugate and a decrease in affinity in the case of albumin conjugate (pH 4.5). At 37°C, the monomeric form was stable for a period up to 48 hours, the same for albumin conjugate (pH 6.8), which indicates that the enzyme is thermally stable in its monomeric form and that conjugation with albumin does not alter this quality.

The pH optimum using maltose as substrate for human placenta- α-glucosidase monomer, albumin conjugate (pH 6.8), albumin conjugate (pH 4.5) and albumin-insulin conjugate are 4, 4, 3 and 4.5 respectively. The Km for the monomer is 0.44 mM and those for albumin conjugate (pH 6.8), albumin conjugate (pH 4.5) and albumin-insulin are 0.66, 0.66 and 0.77 respectively. There seems to be a decrease in affinity in the conjugated forms.

The pH optimum for human placenta- α-glucosidase monomer using glycogen as substrate was 3.5 and for albumin conjugate (pH 6.8) was 3.0 which curve seems to be broader than that of the monomer indicating an increase in stability of the enzyme when conjugated with albumin. The Km for human placenta- α-glucosidase monomer is 0.39 mM while those for albumin conjugate (pH 6.8 and 4.5) are 0.065 and 0.083 mM respectively, which indicates a marked increase in affinity for glycogen for the albumin conjugates. This fact is important because in Pompe's disease, glycogen is the substrate accumulated.

The effect of pH at the time of conjugation on the affinity of α -glucosidases for the substrates are as follows: when conjugated with albumin at pH 6.8 Aspergillus niger- α -glucosidase has a slight decrease in affinity for maltose compared with that for conjugated at pH 4.5. When conjugated with albumin at pH 6.8 A. niger- α-glucosidase has a higher affinity for glycogen compared with the conjugate at pH 4.5. Human placenta- α-glucosidase when conjugated at pH 6.8 and 4.5 shows no difference in affinity for maltose but for glycogen the conjugate at pH 6.8 has a slightly bigger affinity. The effect of pH at the time of conjugation on the pH optimum of α-glucosidase are as follows: when conjugated with albumin at pH 6.8, the pH optimum of A. niger- α -glucosidase increases whereas it decreases when conjugated at pH 4.5 but both broadens the pH activity curve. Thus, the characteristics of enzyme-albumin and enzyme-albumin-insulin conjugates can be manipulated by altering the pH of the reaction medium for the cross-linking step.

The studies so far are not sufficient to determine the therapeutic potential of these enzyme conjugates. However, some conclusions can be drawn. For yeast- α -glucosidase-albumin conjugates the enzyme is not active towards glycogen which is a limitation for its use in the treatment of Pompe's disease where the accumulated substrate is glycogen. However, the enzyme is a good model for some of the positive characteristics of enzyme-albumin polymers such as increased stability at a broad range of pH, increased thermal stability an increased affinity for the substrate (maltose) in the enzyme albumin conjugate. Among the α -glucosidase-albumin conjugates of A. niger prepared at pH

replacement therapy because of its five fold increase in affinity for glycogen though the latter one has a pH optimum closer to the lysosomal pH. α-glucosidase-albumin-insulin polymer also prepared at pH 6.8 retains the high affinity but the thermal stability at 37°C is not as good as that of the albumin conjugate. But the insulin conjugate has a higher targeting efficiency (Poznansky and Singh, 1984). There is the possibility that insulin may give rise to an undesirable effect because insulin normally increases the transport of glucose from blood to liver and muscles where it is largely converted into glycogen. This, in turn, is expected to increase the glycogen content in lysosomes. However, the preliminary clinical trials in our laboratory have shown no marked hypoglycemic effects during the α-glucosidase-albumin-insulin polymer administration. The human placental enzyme also seems to give better polymer when conjugated with albumin at pH 6.8. But the conjugate with A. niger enzyme has a 10 fold higher affinity for glycogen compared with the human enzyme and a pH optimum slightly closer to lysosomal pH.

In addition, A. niger enzyme is easily available in commercial form. On the other hand, the human enzyme is not commercially available and the isolation of the human enzyme in a reasonably pure form and required quantities is a laborious task. Hence, the feasibility of using human enzyme in the near future for replacement therapy seems to be not good unless the molecular cloning technique is developed. But the major disadvantage of the A. niger enzyme is the possibly immunologic reaction. However, A. niger- α-glucosidase-albumin conjugates have been shown to have reduced immunogenicity

that the albumin conjugate of α-glucosidase from A. niger prepared at pH 6.8 is the best choice for enzyme replacement therapy in Pompe's disease.

Finally, it may be mentioned here that further animal experiments and clinical trials are required to evaluate the potential of these enzyme conjugates as therapeutic agents.

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